

## REMARKS

In view of the above amendments and the following remarks, reconsideration of the outstanding office action is respectfully requested. Pursuant to 37 CFR § 1.121, attached as an appendix is a version of the amendments with markings to show changes that have been made.

Interactions between bacterial pathogens and their plant hosts generally fall into two categories: (1) compatible (pathogen-host), leading to intercellular bacterial growth, symptom development, and disease development in the host plant; and (2) incompatible (pathogen-nonhost), resulting in the hypersensitive response, a particular type of incompatible interaction occurring, without progressive disease symptoms. During compatible interactions on host plants, bacterial populations increase dramatically and progressive symptoms occur. During incompatible interactions, bacterial populations do not increase, and progressive symptoms do not occur.

The hypersensitive response is a rapid, localized necrosis that is associated with the active defense of plants against many pathogens. The hypersensitive response elicited by bacteria is readily observed as a tissue collapse if high concentrations ( $\geq 10^7$  cells/ml) of a limited host-range pathogen like *Pseudomonas syringae* or *Erwinia amylovora* are infiltrated into the leaves of nonhost plants (necrosis occurs only in isolated plant cells at lower levels of inoculum). The capacities to elicit the hypersensitive response in a nonhost and be pathogenic in a host appear linked. As noted by Klement, Z., "Hypersensitivity," pages 149-177 in Phytopathogenic Prokaryotes, Vol. 2., M.S. Mount and G.H. Lacy, ed. Academic Press, New York, these pathogens also cause physiologically similar, albeit delayed, necroses in their interactions with compatible hosts. Furthermore, the ability to produce the hypersensitive response or pathogenesis is dependent on a common set of genes, denoted *hrp*. Consequently, the hypersensitive response may hold clues to both the nature of plant defense and the basis for bacterial pathogenicity.

The *hrp* genes are widespread in gram-negative plant pathogens, where they are clustered, conserved, and in some cases interchangeable. Several *hrp* genes encode components of a protein secretion pathway similar to one used by *Yersinia*, *Shigella*, and *Salmonella* spp. to secrete proteins essential in animal diseases. In *E. amylovora*, *P.*

*syringae*, and *P. solanacearum*, hrp genes have been shown to control the production and secretion of glycine-rich, protein elicitors of the hypersensitive response.

The first of these proteins was discovered in *E. amylovora* Ea321, a bacterium that causes fire blight of rosaceous plants, and was designated harpin. Mutations in the encoding *hrpN* gene revealed that harpin is required for *E. amylovora* to elicit a hypersensitive response in nonhost tobacco leaves and incite disease symptoms in highly susceptible pear fruit. The *P. solanacearum* GMI1000 PopA1 protein has similar physical properties and also elicits the hypersensitive response in leaves of tobacco, which is not a host of that strain. However, *P. solanacearum* *popA* mutants still elicit the hypersensitive response in tobacco and incite disease in tomato. Thus, the role of these glycine-rich hypersensitive response elicitors can vary widely among gram-negative plant pathogens.

Other plant pathogenic hypersensitive response elicitors have been isolated, cloned, and sequenced. These include: *Erwinia chrysanthemi*, *Erwinia carotovora*, *Erwinia stewartii*, and *Pseudomonas syringae* pv. *syringae*.

The present invention is a further advance in the effort to identify, clone, and sequence hypersensitive response elicitor proteins or polypeptides from plant pathogens.

In response to the statement on page 2 of the outstanding office action that the drawings were objected to, applicants note that a form PTO 948 did not, in fact, accompany the office action. (The appropriate box on the office action summary was not checked either.) Therefore, applicants cannot meaningfully respond to the statement on page 2.

The title has been changed, thereby overcoming this objection to the application. This objection, too, should be withdrawn.

The rejection of claims 1-16 and 29-31 under 35 U.S.C. § 112, first paragraph, for lack of enablement is rendered moot with respect to claims 11-16 and 29-31 (cancelled without prejudice) and respectfully traversed with respect to claims 1-10.

Applicants have amended claim 1 to recite the hybridization conditions under which a claimed DNA molecule will hybridize to the complement of SEQ. ID. No. 1. Applicants submit that one of ordinary skill in the art can readily perform hybridization protocols using the recited conditions or comparable conditions modified according to one or more known factors affecting hybridization (i.e., Na<sup>+</sup> concentration, temperature, probe size, formamide concentration, etc.).

The invention of claim 1 recites an “isolated DNA molecule encoding a hypersensitive response eliciting protein or polypeptide.” The HR phenomena results from an incompatible interaction between pathogens and their non-host plants. As explained in Gopalan, et. al., “Bacterial Genes Involved in the Elicitation of Hypersensitive Response and Pathogenesis,” Plant Disease 80: 604-10 (1996)(“Gopalan”) (attached hereto as Exhibit 1), this interaction involves a bacterium attempting to infect a host plant, preventing multiplication and spreading of the pathogen, and collapse of plant leaf cells and cell death at the site of infection. This is distinct from a compatible interaction between bacteria and a plant where the bacteria spread in the infected plant, leading to disease symptoms throughout the plant. Id. at 604. Thus, if the protein or polypeptide encoded by the DNA molecule does not elicit a hypersensitive response (i.e., incompatible interaction) when exposed to non-host plants, the encoded protein or polypeptide does not satisfy the limitations of the claimed invention. To determine whether or not a particular protein elicits a hypersensitive response in non-host plants, the protein or polypeptide is applied directly to the plant leaves (see Examples 7 and 14, and Fig. 5A of the present application).

Contrary to the PTO assertion that the present application does not “teach the specific function of the instant gene”, applicants submit that the present application defines a function of the present gene as well as characteristics which are shared among the HrpW protein of the present invention (encoded by claimed DNA molecule of SEQ. ID. No. 1) and other known hypersensitive response elicitors. Beginning at page 27, line 21, it is noted that the HrpW protein is acidic, hydrophilic, glycine-rich, and lacking in cysteine. Moreover, in Example 14, it is reported that the HrpW protein is heat-stable, protease sensitive, and capable of eliciting an HR in tobacco, violet, geranium, tomato, *Kalanchoe diargremontiana*, and *Arabidopsis thaliana* leaves.

It is well recognized in the art that hypersensitive response elicitors have a number of common characteristics. These include their being glycine rich, heat stable, hydrophilic, capable of inducing a hypersensitive response in tobacco after recombinant expression, susceptible to proteolysis, and cysteine lacking. See U. Bonas, “Bacterial Home Goal by Harpins,” Trends Microbiol. 2: 1-2 (1994)(“Bonas I”), attached hereto at Exhibit 2; U. Bonas, “*hrp* Genes of Phytopathogenic Bacteria,” Current Topics in Microbiology and Immunology 192: 79-98 (1994)(“Bonas II”), attached hereto as Exhibit 3; and G. Preston, et. al., “The HrpZ Proteins of *Pseudomonas syringae* pvs. *syringae*, *glycinea*, and *tomato* are Encoded by an Operon Containing *Yersinia ysc* Homologs and Elicit the Hypersensitive

Response in Tomato but not Soybean,” MPMI 8(5): 717-32 (1995)(“Preston”), attached hereto as Exhibit 4.

Moreover, using a 1.4 kb DNA molecule containing SEQ. ID. No. 1 as a probe for high and low stringency Southern hybridization genomic DNA from representative *Erwinia* pathogens, the *hrpW* probe hybridized to genomic DNA from 10 strains of *Erwinia amylovora*, and other species of *Erwinia* including *E. salicis* and *E. caratovora* (Example 10 and Fig. 6). Hybridization was also observed with a clone from *E. chrysanthemi* (Id.).

Thus, one of ordinary skill in the art would be fully able to perform a hybridization experiment using the DNA molecule of SEQ. ID. No. 1, and one of ordinary skill in the art would be fully able to prepare the encoded protein and determine whether the encoded protein does, in fact, elicit a hypersensitive response.

For all of the above reasons, applicants submit that the rejection of claims 1-10 under 35 U.S.C. § 112, first paragraph, for lack of enablement should be withdrawn.

The rejection of claims 1-16 and 29-31 under 35 U.S.C. § 112, first paragraph, for lack of written descriptive support is rendered moot with respect to claims 11-16 and 29-31 (cancelled without prejudice) and respectfully traversed with respect to claims 1-10.

Pursuant to the “Guidelines for Examination of Patent Applications Under the 35 U.S.C. § 112, ¶ 1, ‘Written Description’ Requirement,” 66 Fed. Reg. 1099, 1106 (2001), the written description requirement for a genus can be satisfied by “sufficient description of a representative number of species by actual reduction to practice...or by disclosure of relevant identifying characteristics, i.e., structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between structure and function, or by a combination of such identifying characteristics....” Whether a representative number of species is identified depends on whether the disclosed species represent(s) the entire genus. Id. Applicants submit that these requirements are satisfied by the present application.

In particular, applicants have identified a single representative species which possess the nucleotide sequence of SEQ. ID. No. 1 and encodes the protein of SEQ. ID. No. 2. The HR elicitor protein encoded by this representative species (from *Erwinia amylovora* strain Ea321) is defined in the present application as possessing the following properties: a molecular mass of about 45 kDa (page 9, line 14, which is amended above); an amino acid sequence which is acidic, glycine-rich, and lacks cysteine (page 9, lines 11-14,

which is amended above; Example 11); heat-stability, protease sensitivity, and capability of eliciting an HR in tobacco leaves (page 9, lines 11-14, which is amended above; Example 14); and a C-terminal homology to Pel domains but lacking Pel activity (Examples 10 and 11).

As noted above, it is well established that hypersensitive response elicitors as a class possess similar characteristics, including heat stability, protease sensitivity, high glycine content, substantially no cysteine, etc. as described above (see Bonas I, Bonas II, and Preston). The representative species disclosed in the present application shares these characteristics with other members of the larger class of HR elicitors. Moreover, as noted above, using a DNA molecule of SEQ. ID. No. 1 as a probe for hybridization, the probe hybridized to at least one distinct band for a number of *Erwinia amylovora* strains, as well as several other species of *Erwinia* (Example 17). Applicants indicated that these hybridization results are significant, because they suggest that HrpW exists in several other *Erwinia* species, suggesting a role for HrpW in pathogenesis (see page 30, lines 26-30). Thus, applicants demonstrated that the single demonstrated species possesses a combination of identifying characteristics which are representative of the protein encoded by DNA molecules of the presently claimed genus. Moreover, the demonstration of hybridization with DNA from other pathogens indicates that *hrpW* is conserved at least among various pathogenic *Erwinia* species. One of ordinary skill in the art clearly would recognize that applicants were in possession of the presently claimed genus.

The PTO has provided no evidentiary basis to believe that the single disclosed species is not representative of the claimed genus and, therefore, the rejection of claims 1-10 for lack of written descriptive support should be withdrawn.

The rejection of claims 1-16 and 29-31 under 35 U.S.C. § 112 (second paragraph) for indefiniteness is rendered moot with respect to claims 11-16 and 29-31 (cancelled without prejudice) and respectfully traversed with respect to claims 1-10.

The rejection of claims 1-10 under 35 U.S.C. § 102(a) as being anticipated by either Kim et al., "Hrp-secreted proteins and avirulence protein homologs of *Erwinia amylovora*," Phytopathol. 87:S52 (1997) ("Kim I") or Kim et al., "HrpW, A New Harpin of *Erwinia amylovora*, is a Member of a Family of Pectate Lyases," Phytopathol. 87:S52 (1997)

("Kim II") is respectfully traversed. (Because it is unclear whether the PTO is citing to Kim I or Kim II in making the rejection, applicants have addressed them both below.)

Kim I simply identifies the genomic location of the *Erwinia amylovora hrpW* gene as being between the *hrp* region and the *dsp* region. Kim I does not disclose how one of ordinary skill in the art would go about isolating the *hrpW* gene let alone its nucleic acid sequence (e.g., SEQ. ID. No. 1). Therefore, Kim I cannot have anticipated the presently claimed invention.

Kim II reports the identification of the *hrpW* gene in *Erwinia amylovora* and the fact that the HrpW protein shares the common features shared by other hypersensitive response elicitors and appears to be conserved among *Erwinia*. Kim II also reports the unique property of the HrpW homology to pectate lyases, although acknowledging that HrpW does not possess such activity. No sequence data was reported.

As provided in the accompanying Declaration of Steven V. Beer Under 37 C.F.R. § 1.132, Dr. Beer states that contributions made by Ms. Zumoff to the Kim II publication involved her role as a lab technician (¶ 4). Ms. Zumoff did not provide any contribution to the conception of the invention as described and claimed in the above-identified application (Id.). Thus, Kim II is not available as prior art under 35 U.S.C. 102(a). See In re Katz, 687 F.2d 450 (Fed. Cir. 1982).

For all of the above reasons, the rejection of claims 1-10 over either Kim I or Kim II is improper and should be withdrawn.

The rejection of claims 1-16 and 29-31 under 35 U.S.C. § 102(e) as being anticipated by U.S. Patent No. 5,850,015 to Bauer et al. ("Bauer '015 patent") is rendered moot with respect to claims 11-16 and 29-31 (now cancelled without prejudice) and respectfully traversed with respect to claims 1-10. The PTO cites to the Bauer '015 patent the proposition that the *hrpN<sub>Ech</sub>* gene would share at least one nucleotide with SEQ. ID. No. 1 and its encoded protein would share at least one amino acid with the protein of SEQ. ID. No. 2. Applicants submit that the PTO's position is obviated by the above amendments, because the Bauer '015 patent does not teach a DNA molecule as recited in claim 1. Therefore, the rejection of claims 1-10 should be withdrawn.


The rejection of claims 1-10 under 35 U.S.C. § 102(b) as being anticipated by Wei et al., "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen

*Erwinia amylovora*," Science 257:85-88 (1992) ("Wei") is respectfully traversed. The PTO cites to Wei for the proposition that the *hrpN* gene would share at least one nucleotide with SEQ. ID. No. 1 and its encoded protein would share at least one amino acid with the protein of SEQ. ID. No. 2. Applicants submit that the PTO's position is obviated by the above amendments, because Wei does not teach a DNA molecule as recited in claim 1. Therefore, the rejection of claims 1-10 should be withdrawn.


In view of the all of the foregoing, applicants submit that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

Date: March 22, 2002

  
Edwin V. Merkel  
Registration No. 40,087

NIXON PEABODY LLP  
Clinton Square, P.O. Box 31051  
Rochester, New York 14603-1051  
Telephone: (585) 263-1128  
Facsimile: (585) 263-1600

Certificate of Mailing - 37 CFR 1.8(a)	
I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, Washington, D.C. 20231, on the date below.	
Date: <u>3/22/02</u>	 Wendy L. Harrold

**Appendix**  
**Version With Markings to Show Changes Made**  
**Page 1 of 2**

In reference to the amendments made herein to the specification and claims, additions appear as underlined text while deletions appear as strikeout text, as indicated below:

In the Specification:

At page 5, lines 12-15:

Figure 2A shows the expression of *hrpW* by a T7 RNA polymerase-directed gene expression system. Lanes 1, *E. coli*DH5 $\alpha$ (pGP1-2/pBC SK (-)); 2, *E. coli*DH5 $\alpha$ (pGP1-2/pCPP1232). The arrow between 84kD and 53kD points to the band in lane 2 corresponding to the HrpW protein.

At page 9, lines 11-14:

This protein or polypeptide is acidic, rich in glycine and serine, and lacks cysteine. It is also heat stable, protease sensitive, and suppressed by inhibitors of plant metabolism. The protein or polypeptide of the present invention has a predicted molecular size of ca. ~~4-5~~ 45 kDa.

In the Claims:

Claims 1-7 and 9-10 have been amended as follows:

1. (Amended) An isolated DNA molecule encoding a hypersensitive response eliciting protein or polypeptide, wherein the isolated DNA molecule is selected from the group consisting of (a) a DNA molecule comprising ~~a nucleotide sequence of~~ SEQ. ID. No. 1, (b) a DNA molecule encoding a protein comprising ~~an amino acid of~~ SEQ. ID. No. 2, (c) a DNA molecule which hybridizes to a DNA molecule comprising ~~a nucleotide sequence~~ the complement of SEQ. ID. No. 1 under ~~stringent~~ conditions comprising hybridization at a temperature of about 65°C in a hybridization medium comprising about 1M NaCl, and (d) a DNA molecule complementary to DNA molecules (a), (b), ~~and~~ or (c).

2. (Amended) An isolated DNA molecule according to claim 1, wherein said DNA molecule is a DNA molecule comprising ~~a nucleotide sequence of~~ SEQ. ID. No. 1.



**Appendix**  
**Version With Markings to Show Changes Made**  
**Page 2 of 2**

3. (Amended) An isolated DNA molecule according to claim 1, wherein said DNA molecule is a DNA molecule encoding protein comprising ~~an amino acid of~~ SEQ. ID. No. 2.

4. (Amended) An isolated DNA molecule according to claim 1, wherein said DNA molecule is a DNA molecule which hybridizes to a DNA molecule comprising ~~a nucleotide sequence~~ the complement of SEQ. ID. No. 1 under ~~stringent~~ conditions comprising hybridization at a temperature of about 65°C in a hybridization medium comprising about 1M NaCl.

5. (Amended) An isolated DNA molecule according to claim 1, wherein said DNA molecule is a DNA molecule complementary to DNA molecules (a), (b), ~~and~~ or (c).

6. (Amended) An expression vector ~~transformed with~~ comprising the DNA molecule of claim 1.

7. (Amended) An expression vector according to claim 6, wherein the DNA molecule is in ~~proper~~ sense orientation ~~and correct reading frame~~.

9. (Amended) A host cell according to claim 8, wherein the host cell ~~is selected from the group consisting of~~ a plant cell or a bacterial cell.

10. (Amended) A host cell according to claim 8, wherein the DNA molecule is ~~transformed with~~ comprised within an expression vector.

# Bacterial Genes Involved in the Elicitation of Hypersensitive Response and Pathogenesis

Intensive molecular genetic studies undertaken in the past 10 years have started to solve many of the puzzles in the area of compatibility and incompatibility between plants and bacterial pathogens. These studies have provided answers to some of the most fundamental questions in plant pathology: What bacterial genes are involved in the establishment of compatibility or incompatibility between plants and necrogenic bacteria? What traits distinguish plant-pathogenic bacteria from saprophytic bacteria? Are these genes and traits common in seemingly very diverse groups of plant-pathogenic bacteria, from soft-rot *Erwinias* to local lesion-forming *Pseudomonads*? In this article, we will discuss some recent advances in understanding the compatibility or incompatibility between plants and necrogenic bacteria (bacteria that cause tissue necrosis). The potential application of these advances to disease management will be addressed briefly. Interested readers should consult other recent reviews (6,8,45,50) for a more technical discussion on this topic.

## Plant-Bacteria Interactions: Incompatible vs. Compatible

Plant-pathogenic bacteria cause devastating diseases on many important crop plants. Some bacteria, such as *Agrobacterium tumefaciens*, cause tissue deformation (tumors) by altering hormone balance in infected plant tissues. Other bacteria cause wilt or soft rot by interfering with the function of the plant vascular system or by disintegrating plant tissues, respectively. Many pathovars of *Pseudomonas syringae* and *Xanthomonas campestris* cause local lesions on various plant tissues. Disease symptoms caused by most plant-pathogenic bacteria involve plant cell death. In this article, only necrogenic bacteria will be

discussed. Therefore, gall-forming *A. tumefaciens* and other bacteria that do not cause necrosis will not be addressed.

Plant-bacteria interactions can be generally classified as compatible or incompatible interactions. In a compatible interaction, a susceptible host plant is infected by a virulent (or compatible) bacterium, resulting in the multiplication and spread of the bacterium in infected plant tissues and the appearance of disease symptoms. In an incompatible interaction, an avirulent (or incompatible) bacterium attempts to infect a resistant host plant or a nonhost plant, but the multiplication and spread of the bacterium are severely restricted. A hallmark of many incompatible interactions is the occurrence of rapid plant cell death at or near the attempted infection site, a phenomenon known as the hypersensitive response (HR; 16,29). That is, although an avirulent bacterium is unable to cause typical spreading disease symptoms in a resistant host or nonhost plant, it is able to elicit localized plant cell death. The HR is associated with a wide array of defense responses that may inhibit further pathogen invasion, including synthesis of antimicrobial compounds, induction of plant defense genes, and strengthening of the plant cell wall by rapid cross-linking of cell wall components (10,32).

Although a true plant-pathogenic bacterium can elicit a dramatic plant response—either disease or resistance—in a healthy plant with the appropriate genetic background, saprophytic bacteria or bacteria that are pathogenic on organisms other than higher plants are generally unable to initiate any interactions in plants. Of 1,600 known species of bacteria, only about 80 species have been found to cause plant disease (1). What are the features that distinguish plant-pathogenic bacteria from other types of bacteria? Taxonomic differences give no clue to the differences in pathogenicity. For example, *Erwinia amylovora*, the bacterium that causes fire blight, is taxonomically more closely related to the human pathogen *Escherichia coli* and *Yersinia* spp. than to another common plant pathogen, *P. syringae*.

## Genes Controlling Compatibility Between Plants and Bacteria

In the early 1980s, a number of researchers started to use transposon-mediated mutagenesis, a technique developed in the study of *E. coli*, to reveal bacterial genes that play important roles in various plant-bacteria interactions. A transposon is a mobile DNA element that can hop in and out of the bacterial chromosome. When a transposon hops into a gene on the chromosome, the gene is physically disrupted and cannot produce a functional product (Fig. 1). If the gene happens to be important in plant-bacterial interactions, the mutant bacterium carrying the disrupted gene will show a defect in initiating normal plant-bacterial interactions.

Using such a mutagenesis technique, Niepold et al. (35) and Lindgren et al. (33) identified clusters of bacterial genes, known as *hrp* (for HR and pathogenicity) genes, in the bean pathogens *Pseudomonas syringae* pv. *syringae* and *P. s.* pv. *phaseolicola*, respectively. Transposon-induced mutations in *hrp* genes were found to abolish the ability of *P. syringae* to elicit the HR in nonhost plants or to cause disease in host plants (33,35). *hrp* mutants behave very much like bacteria that have no apparent interactions with plants, such as *E. coli*. The identification of *hrp* genes suggested that the molecular mechanism(s) underlying bacterial pathogenicity and bacterial elicitation of plant disease resistance may involve the same bacterial genes.

*hrp* genes have been isolated from many plant-pathogenic bacteria and have been characterized most extensively from *P. s.* pv. *syringae*, *P. s.* pv. *phaseolicola*, *Pseudomonas solanacearum* (which causes wilt in many solanaceous plants), *Xanthomonas campestris* pv. *vesicatoria* (which causes bacterial spot on tomato and pepper), and *E. amylovora* (6,8,45). Isolation (cloning) of *hrp* genes was accomplished by inserting random genomic DNA fragments from a wild-type, plant-pathogenic bacterium into a cloning vector, followed by introduction of cloned DNA fragments into *hrp* mutants

Dr. He's address is: MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824-1312; Phone: 517-353-9181; Fax: 517-353-9168; E-mail: hes\_pilot@msu.edu

(Fig. 1). If a cloned DNA fragment carries a wild-type copy of the mutated *hrp* gene in an *hrp* mutant, it will produce a functional *hrp* gene product and therefore complement the mutated *hrp* gene located in the chromosome (Fig. 1). Surprisingly, the cloned *hrp* clusters from *P. s. pv. syringae* 61 and *E. amylovora* 321 enabled nonpathogens (e.g., *E. coli* or *Pseudomonas fluorescens*) to elicit the HR in plants (5,24). The functional cloning of these two *hrp* clusters in *E. coli* revealed that the minimum number of genes required for elicitation of the HR by plant-pathogenic bacteria is carried on a DNA fragment about 25 to 30 kb in length, a very small portion of the bacterial genome (which is normally about 4,000 to 5,000 kb).

DNA-DNA hybridization studies indicate that at least some *hrp* genes are similar among necrogenic bacteria belonging to different genera (*P. syringae*, *E. amylovora*, *Erwinia stewartii*, *P. solanacearum*, and *X. campestris*) (31). Recent DNA sequence studies confirm that many *hrp* genes cloned from diverse plant-pathogenic bacteria are homologous (23,46). Thus, *hrp* genes appear to be universal among diverse necrosis-causing, gram-negative bacterial pathogens of plants.

### Biochemical Functions of *hrp* Genes

The biochemical functions of *hrp* genes have remained a puzzle until recently. DNA sequencing has played a major role in the determination of many *hrp* gene functions. As will be discussed, many *hrp* genes have striking similarities with genes of known function. Figure 2 shows the gene organization and likely functions of *hrp* genes of *P. s. pv. syringae* (23). There are at least 25 *hrp* genes in this bacterium. Based on DNA sequence similarity to other known genes and subsequent biochemical and molecular characterization, we now know that *hrp* genes have at least three biochemical functions: gene regulation, protein secretion, and production of HR elicitor proteins.

**1. Gene regulation.** It was discovered that *hrp* genes either are not expressed or are expressed at very low levels (i.e., they make very low levels of protein products) when bacteria were grown in nutrient-rich bacteriological media, whereas they are highly expressed when bacteria are in the intercellular space (apoplast) of plant tissues (25,37,41,46,48,52,53). What conditions in plant tissues induce the expression of *hrp* genes, and how do bacteria detect these inducing conditions? Unlike viruses, nematodes, and many fungi, plant-pathogenic bacteria do not invade living plant cells. Therefore, signal exchanges between plant cells and bacteria must occur in (or through) the apoplast outside the plant cell. A number of laboratories have observed that induction of *P. syringae* *hrp* genes could be achieved by using artificial

minimal media lacking complex nitrogen nutrients, indicating that lack of nutrients in the plant apoplast may be the signal for the induction of *hrp* genes (25,37,52,53). Specific compounds (e.g., sucrose and sulfur-containing amino acids) present in the plant apoplast may also serve as signals for the induction of *X. c. pv. vesicatoria* *hrp* genes (41). The induction of *hrp* genes in the nutrient-poor plant apoplast or in artificial minimal media indicates that *hrp* genes may be involved in bacterial nutrition in plants.

How do bacteria sense the plant apoplast environment? It was found that at least three of the 25 *hrp* gene products are involved in detection of the apoplast environment by *P. syringae*: *hrpL*, *hrpS*, and *hrpR* (18,51; Fig. 2). The *hrpS* and *hrpR* are among the first two *hrp* genes to be expressed once bacteria enter plant tissues (51,52). It has been hypothesized that the *HrpS* and *HrpR* proteins, once produced, bind to the "promoter" sequence of the *hrpL* gene to "promote" the production of the *HrpL* protein (51). Once the *HrpL* protein is produced, it activates promoters of other *hrp* genes and some bacterial avirulence (*avr*) genes, which determine gene-for-gene interactions between bacteria and plants (25,26,38,40,51; Fig. 3). Not all bacterial *avr* genes are regulated by *hrp* genes (30). Interestingly, *hrpS* and *hrpR*

are similar in sequence to a family of bacterial proteins that regulate genes involved in diverse metabolic functions, including genes involved in nutrient transport and metabolism (18,51). The sequence similarity of *hrpS* and *hrpR* with gene regulators involved in nutrition appears to support the hypothesis that *hrp* genes are involved in bacterial nutrition in the nutrient-poor plant apoplast. This hypothesis is further supported by the observation that the expression of *hrp* genes can be turned off by complex nitrogen sources, tricarboxylic acid cycle intermediates, high osmolarity, and neutral pH, some of which are characteristic of rich bacterial media (25,37,41,46,52,53).

An *hrpS* homolog has been found in a very different bacterium, *E. amylovora* (S. V. Beer, personal communication). In *P. solanacearum*, a different *hrp* gene (*hrpB*) was found to be involved in the detection of the plant apoplast (15). Thus, different bacteria may or may not use the same mechanism to detect the apparently similar environment in the plant apoplast.

**2. Protein secretion.** One surprising finding from the sequence analysis of *hrp* genes was that many *hrp* genes show striking similarities to those involved in the secretion of proteinaceous virulence factors in human and animal pathogenic bacteria (12,17,22,39,46). Most plant-pathogenic

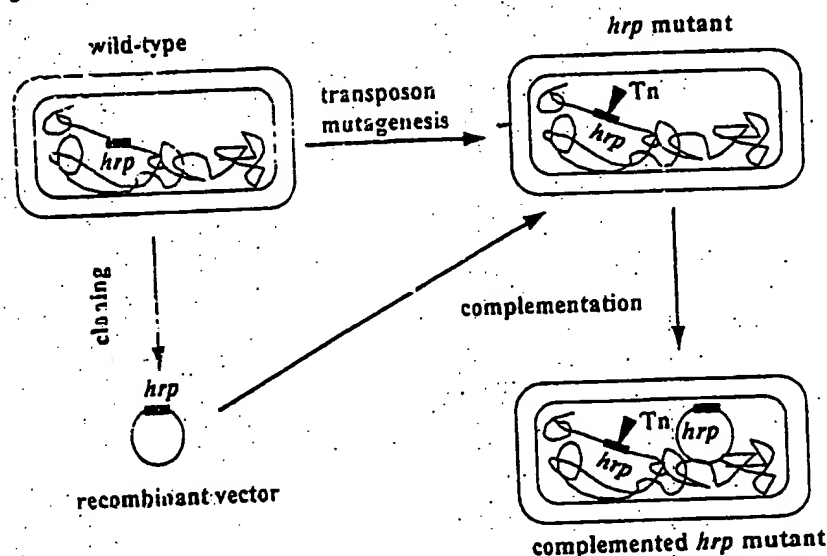


Fig. 1. Diagram of molecular techniques commonly used in the cloning of *hrp* genes. A wild-type bacterium is mutagenized by random insertion of a transposon (Tn) into its genome. When a transposon inserts into a wild-type *hrp* gene (in red), it physically disrupts the *hrp* gene (in green). The transposon-inserted *hrp* gene cannot produce a functional product, and the resulting bacterium is called a *hrp* mutant. The *hrp* mutant can no longer induce the hypersensitive response (HR) in resistant plants or cause disease in susceptible plants. To isolate (clone) the *hrp* gene identified by transposon mutagenesis, a gene library is established by inserting pieces of the wild-type genomic DNA into a cloning vector (indicated by a circle). The vector carrying foreign DNA inserts (recombinant vector) is then introduced into the *hrp* mutant. If a recombinant vector happens to carry a wild-type copy of the mutated *hrp* gene, it will produce a functional *hrp* gene product lacking in the *hrp* mutant, thus recovering the ability of the mutant to induce the HR in resistant plants and to cause disease in susceptible plants. The *hrp* mutant phenotype is therefore complemented by this recombinant vector.

bacteria that cause necrosis are gram-negative, that is, they have two cell membranes enveloping the cytoplasm. For any large molecule (e.g., a protein) to go through a lipid membrane, a special secretion apparatus or channel composed of many proteins must be assembled across both cell membranes. Gram-negative plant pathogenic bacteria are known to make several types of secretion apparatus. For example, *Erwinia chrysanthemi*, a bacterium that causes soft rot, makes one type of secretion apparatus for proteases and another for plant cell wall-degrading enzymes (21,39). Both types of secretion apparatus are widely conserved among many other bacteria, including human pathogens such as *E. coli* and *Pseudomonas aeruginosa* (21,39). The *hrp* genes were found to specify a third type of secretion apparatus, the Hrp secretion apparatus, which appears to be similar to the one discovered in several human-pathogenic bacteria, including *Yersinia* spp. (12,17,22,39,46). Interestingly, although the regulatory *hrp* genes in different bacteria may be different (*hrpS*, *hrpR*, and *hrpL* in *P. syringae* versus *hrpB* in *P. solanacearum*), most *hrp* genes involved in the assembly of the Hrp secretion apparatus are similar among diverse plant-pathogenic bacteria. This suggests that although different bacteria may detect the plant apoplast environment in their own unique ways, they nevertheless produce similar types of protein secretion apparatus.

3. Production of elicitor proteins. The discovery of the novel Hrp secretion appa-

atus raised an immediate question: What are the proteins that traverse it? Since *hrp* genes are essential for bacteria, both to elicit the plant HR and to cause disease, it was expected that some of the proteins that traverse the Hrp secretion apparatus may be elicitors of plant HR and that others may be involved in causing necrosis during pathogenesis. Wei et al. (47) first provided evidence that one of the *E. amylovora* *hrp* genes (*hrpN*) encodes a proteinaceous elicitor (harpin). Harpin elicits HR necrosis when injected into the apoplast of appropriate plants (47). Although no *hrpN* gene homolog could be found in *P. syringae*, another proteinaceous HR elicitor (harpin<sub>ps</sub>) was identified and was shown to be encoded by a different *hrp* gene, *hrpZ* (20,36). Furthermore, harpin<sub>ps</sub> was the first extracellular protein shown to be secreted via the Hrp secretion apparatus (20). A third bacterial protein elicitor of the HR was identified in *P. solanacearum* and was named PopA1 (2). The *E. amylovora* harpin, *P. s. pv. syringae* 61 harpin<sub>ps</sub>, and *P. solanacearum* PopA1, although largely dissimilar in primary sequences, share similar properties that may be important in their HR elicitor activities. They are all heat stable, glycine rich, and hydrophilic. Homologs of *E. amylovora* harpin and *P. s. pv. syringae* 61 harpin<sub>ps</sub> have been identified in other pathogenic strains that belong to the genus *Erwinia* and the species *P. syringae*, respectively (4,20). Thus, at least three proteins that traverse the Hrp secretion apparatus of three diverse bacteria elicit the HR.

## The Search for Proteins that Traverse the Hrp Apparatus

As mentioned earlier, bacterial mutants defective in the Hrp secretion apparatus are unable to elicit the HR in resistant plants and to cause disease in susceptible plants. The question is, how many proteins are secreted via the Hrp secretion apparatus? If harpins and PopA are the only proteins that traverse the Hrp secretion apparatus, then mutations in the genes that make harpins and PopA would also eliminate the ability of bacteria to elicit the HR in resistant plants and to cause disease in host plants. However, if there are other pathogenicity- or HR-related proteins secreted via the Hrp apparatus, mutations in only harpin- or PopA-encoding genes would not completely abolish the ability of bacteria to elicit the HR in resistant plants or to cause disease in host plants. Wei et al. (47) reported that mutations in the gene coding for harpin of *E. amylovora* destroyed the ability of the bacteria both to trigger the HR in resistant nonhost tobacco and to cause disease in susceptible pear fruits. Mutations in the gene coding for harpin<sub>ps</sub> of *E. chrysanthemi* prevented the bacterium from triggering the HR in the nonhost tobacco and reduced the ability of the bacterium to initiate disease lesions in host plants (4). In the case of harpin<sub>ps</sub> of *P. syringae*, mutation analysis has been complicated by the complex gene structure and organization surrounding the *hrpZ* gene. Conclusive data regarding the role of harpin<sub>ps</sub> in plant-*P. syringae* interactions are yet to be shown. PopA1 was shown to

## *Pseudomonas syringae* *hrp* gene cluster

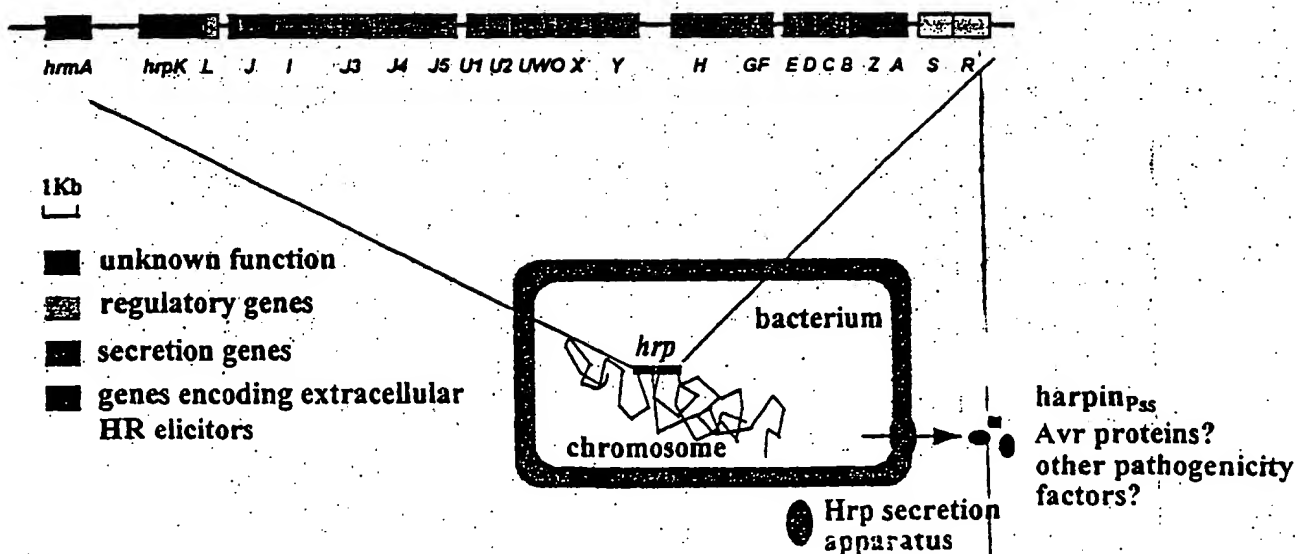


Fig. 2. *hrp* genes of *Pseudomonas syringae*. There are at least 25 *hrp* genes (*hrpA* to *hrpZ*) in *P. syringae*. *hrpS*, *hrpR*, and *hrpL* (in yellow) are involved in the detection of the plant apoplast environment and in the activation of all other *hrp* genes, *avr* genes, and possibly other pathogenicity-related genes. Most other *hrp* genes (in red) are involved in the assembly of the Hrp secretion apparatus in the bacterial envelope, through which travels a newly discovered class of bacterial virulence/avirulence proteins (in green), including HrpZ.

be dispensable for pathogenicity of *P. solanacearum* in the susceptible host plant, tomato, or for HR elicitation in the nonhost plant, tobacco (2), indicating that there must be other HR-elicitors and pathogenicity factors that traverse the Hrp secretion apparatus in this bacterium. Further examination indicated that PopA1 may function as an avirulence gene, mediating gene-for-gene interaction in certain *Penunia-P. solanacearum* interactions (2,45). Thus, the Hrp secretion apparatus in each bacterium may secrete a different number of proteins. Identification of other proteins that traverse the Hrp secretion apparatus is now an active research area and is essential for a complete understanding of *hrp*-mediated plant-bacterial interactions.

#### Factors Modifying *hrp* Gene-Mediated Compatibility

Two broad classes of bacterial genes may superimpose their functions on the *hrp* gene-mediated compatibility or incompatibility between plants and bacteria: *avr* genes and various virulence genes. The *avr* genes mediate genotype-specific incompatibility in resistant host plants. Virulence genes promote the production of disease symptoms and are usually needed for the full virulence of bacteria.

#### Bacterial *avr* Genes

Flor (14) formulated the gene-for-gene hypothesis in his work on flax-flax rust interactions. Flor hypothesized that the resistance of a given flax cultivar to a given fungal race is the result of the interaction between a fungal *avr* gene and a corresponding flax resistance gene. Therefore, the interactions between the plant's resistance genes and the pathogen's *avr* genes would limit the host range of the pathogen. Staskiewicz et al. (44) first cloned an *avr* gene from a soybean bacterial pathogen, *Pseudomonas syringae* pv. *glycinea*, and showed that the cloned *avr* gene could convert virulent *P. s.* pv. *glycinea* strains that cause disease into avirulent strains that elicit the HR in certain soybean cultivars carrying the corresponding resistance genes, thus validating the role of *avr* genes in controlling host range. Since then, numerous *avr* genes have been cloned from plant-pathogenic bacteria (27). Several plant resistance genes have also been cloned using molecular genetic approaches (e.g., 34,43).

What is the relationship between the *avr* genes and *hrp* genes, both of which are involved in eliciting the HR? Several laboratories have observed that *avr* genes cannot trigger the genotype-specific HR in *hrp* mutants, i.e., *avr* genes depend on functional *hrp* genes for expressing their phenotype (25,26,28,38,40). There are several ways of explaining such dependence (Fig. 4). One possibility is that Avr proteins are dependent on the Hrp secretion apparatus for secretion. Alternatively, Avr function requires a prior plant response

elicited by the *hrp*-controlled extracellular factors (such as harpins). A third possibility is that Avr proteins, with no HR-eliciting activity by themselves, cause the cultivar-specific HR by either covalently modifying harpins or modulating the expression of harpins in a plant resistance gene-dependent manner yet to be understood. Finally, it is also possible that Avr proteins are secreted directly into the plant cell with the help of harpins, assuming that receptors for Avr proteins are inside the plant cell. Studies are being carried out to resolve these possibilities.

#### Bacterial Virulence Factors

The genetic diversity of plant-pathogenic bacteria is reflected in their ability to cause diverse disease symptoms ranging from soft rot to tissue necrosis to "wildfire." These diverse disease symptoms are likely the result of the action of several, sometimes unique, virulence factors produced by a given bacterium in addition to *hrp*-controlled pathogenicity

factors. For example, research from many laboratories has shown that toxin production plays an important role in the formation of chlorosis and necrosis (3,19,49). Extracellular polysaccharides may be involved in the formation of water-soaking lesions (11,13) and in the production of wilt symptoms by clogging the plant vascular system (9). Plant cell wall-degrading enzymes are responsible for tissue disintegration and the appearance of the soft-rot symptom (7). Plant hormones produced by plant-pathogenic bacteria are involved in the induction of tissue deformation (42).

Both *hrp* genes and bacterial virulence factors are necessary for disease symptom production, but what is the relationship between them? A logical relationship would be that *hrp*-controlled extracellular factors are involved in obtaining nutrients in early stages of pathogenesis, whereas other virulence factors drive the initial compatible stage into a fully compatible one, leading to the production of various disease symptoms. At least two lines of

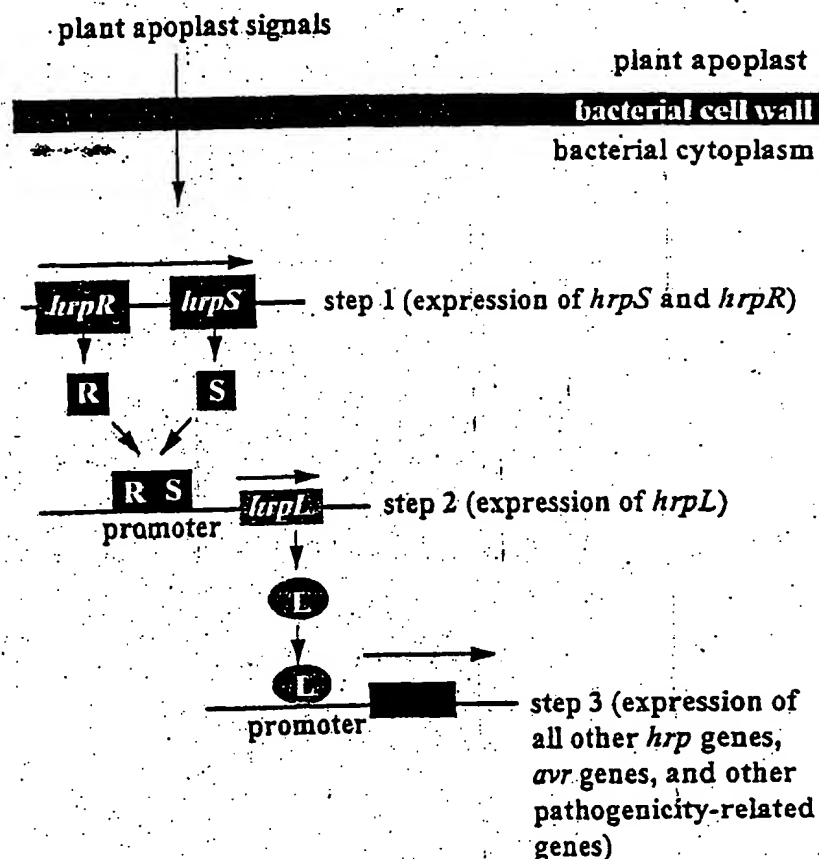


Fig. 3. Diagram of the signal transduction cascade in the detection of the plant apoplast environment by *Pseudomonas syringae*. The plant apoplast environment (limited nutrients and/or certain unique compounds) activates the expression of *hrpS* and *hrpR* by a mechanism yet to be understood (step 1). The *hrpS* and *hrpR* gene products (S and R, respectively) bind to and activate the promoter of the *hrpL* gene (step 2). The *hrpL* gene product (L), in turn, binds to promoters of other *hrp* genes, *avr* genes, and other bacterial pathogenicity-related genes to promote the expression of these genes, resulting in the initiation of diverse plant-bacteria interactions (step 3). Modified from Xiao et al. (51).

evidence seem to support this relationship. First, *hrp* genes are highly conserved among diverse plant-pathogenic bacteria, whereas virulence factors vary greatly among bacteria. Second, while mutations in the *hrp* gene completely abolish both bacterial pathogenicity and elicitation of the HR, mutations in virulence genes (e.g., toxin-production genes) often do not eliminate pathogenicity and have no effect on bacterial elicitation of the HR (3,19,49).

### *hrp* Gene Functions and Disease Management

A major reason for discovering bacterial and plant factors critical for bacterial pathogenesis and plant resistance is to develop novel and environmentally safe strategies for controlling plant diseases. The discovery that the Hrp secretion apparatus is crucial to bacterial pathogenesis provides a foundation for designing novel chemicals and antibodies that would block

the assembly of the Hrp secretion apparatus or the passage of bacterial virulence proteins through it. Alternatively, susceptible crop plants could be genetically engineered with genes encoding proteinaceous HR elicitors, such as harpins, under the control of plant promoters inducible by virulent pathogens. If this approach were successful, the HR or resistance would be triggered in otherwise compatible interactions, limiting disease development.

### Acknowledgments

We thank Karen Bird for help in preparation of this paper, Paul Vincelli and Robert P. Scheffer for critical review, and Marlene Cameron for illustrations. Research in our laboratory is supported by grants from USDA and DOE.

### Literature Cited

1. Agrios, G. N. 1988. Plant Pathology. 3rd ed. Academic Press, San Diego, CA.
2. Ariat, M., van Gijsegem, P., Heut, J. C., Pernollet, J. C., and Boucher, C. A. 1994. PopA1, a protein which induces a hypersensitivity-like response on specific *Pennisia* genotypes, is secreted via the Hrp pathway of *Pseudomonas solanacearum*. EMBO J. 13:543-553.
3. Barta, T. M., Kinscherf, T. G., and Willis, D. K. 1992. Regulation of tabtoxin production by the *temA* gene in *Pseudomonas syringae*. J. Bacteriol. 174:3021-3029.
4. Bauer, D. W., Wei, Z.-M., Beer, S. V., and Collmer, A. 1995. *Erwinia chrysanthemi* harpin<sub>ca</sub>: An elicitor of the hypersensitive response that contributes to soft-rot pathogenesis. Mol. Plant-Microbe Interact. 8:484-491.
5. Beer, S. V., Bauer, D. W., Jiang, X. H., Laby, R. J., Soeath, B. J., Wei, Z., Wilcox, D. A., and Zamoff, C. H. 1990. The *hrp* gene cluster of *Erwinia amylovora*. Pages 53-60 in: Advances in Molecular Genetics of Plant-Mi-

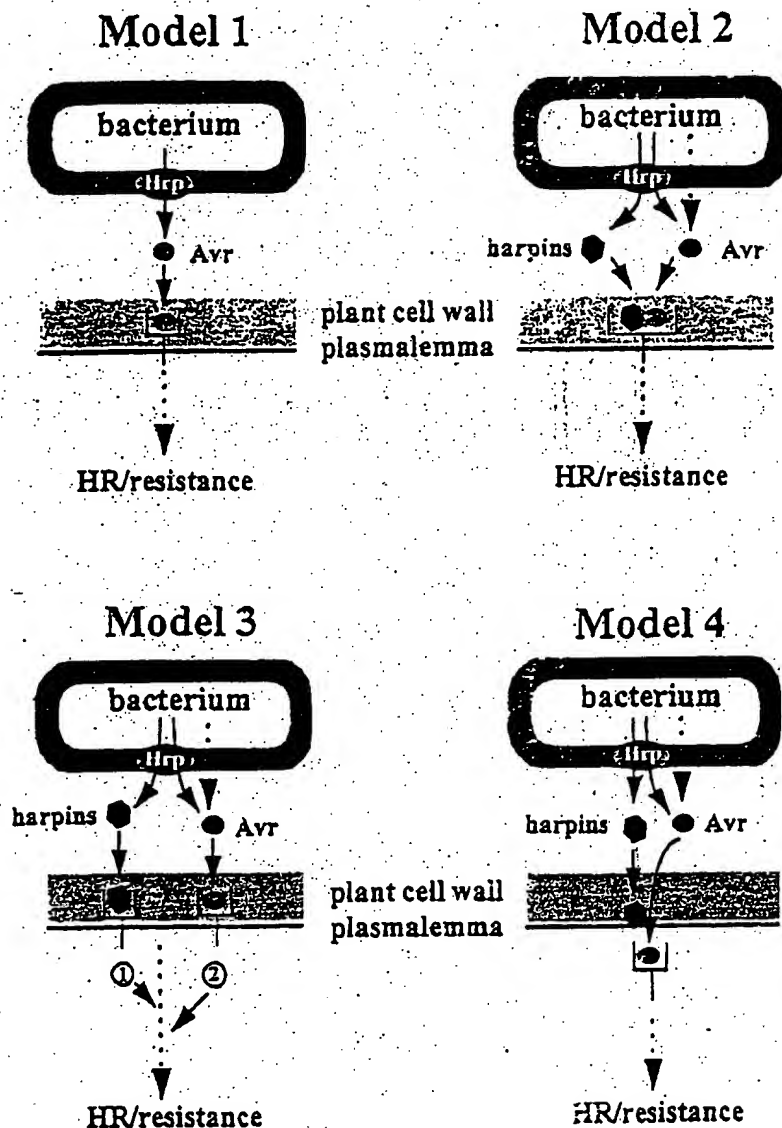


Fig. 4. Working models for possible interactions between *hrp* genes and *avr* genes. Model 1: Avr signals (Avr proteins or their enzymatic products) are secreted through the Hrp secretion apparatus to elicit the hypersensitive response (HR) and resistance. Model 2: Harpins and Avr signals modify each other before interacting with plant receptors. Avr signals may or may not be secreted via the Hrp secretion apparatus. Model 3: Harpins and Avr signals interact with respective plant receptors. Plant response elicited by harpins must precede plant response elicited by Avr signals. Avr signals may or may not be secreted via the Hrp secretion apparatus. Model 4: Avr proteins are secreted into the plant cell with the help of harpins. Avr signals may or may not be secreted via the Hrp secretion apparatus. In models 1 to 3, receptors for Avr proteins are presumed to be on the plant cell surface. In model 4, receptors for Avr proteins are inside the plant cell.

## PLANT-PARASITIC NEMATODES

A Pictorial Key to Genera. 5th Edition

WILLIAM F. MAI & PETER G. MULLIN

WITH HOWARD H. LYON & KENT LOEFFLER

"This [edition] will be welcomed by both students and practitioners. . . . The illustrations alone place this key in a class of its own."—JOHN M. FERRIS, Purdue University

Formerly titled *Pictorial Key to Genera of Plant-Parasitic Nematodes*, this volume has been the standard work on plant disease around the globe. Now in its fifth edition, it remains a comprehensive key to plant-parasitic nematodes and the only guide to feature both photographs and drawings.

A CORSTOCK BOOK

34 b&w photos, 44 drawings \$49.95

At bookstores, or call (607) 277-2211

CORNELL UNIVERSITY PRESS

SAGE HOUSE • 512 E. STATE ST. • ITHACA NY 14850

- crobe interactions, Vol. 1. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht.
6. Bonas, U. 1994. *hrp* genes of phytopathogenic bacteria. *Curr. Top. Microbiol. Immunol.* 192:79-98.
  7. Collmer, A., and Keen, N. T. 1986. The role of pectic enzymes in plant pathogenesis. *Annu. Rev. Phytopathol.* 24:383-409.
  8. Dangl, J. L. 1995. Genes involved in bacterial pathogenesis of plants. Pages 293-304 in: *Pathogenesis and Host Specificity in Plant Diseases: Histochemical, Biochemical, Genetic and Molecular Bases*. Vol. 1. Prokaryotes. U. S. Singh, R. P. Singh, and K. Kohmoto, eds. Pergamon, U.K.
  9. Denny, T. P. 1995. Involvement of bacterial polysaccharide in plant pathogenesis. *Annu. Rev. Phytopathol.* 33:173-179.
  10. Dixon, R. A., Harrison, M. J., and Lamb, C. J. 1994. Early events in the activation of plant defense responses. *Annu. Rev. Phytopathol.* 32:479-501.
  11. El-Banoby, F. E., and Rudolph, K. 1979. Induction of water-soaking in plant leaves by extracellular polysaccharides from phytopathogenic pseudomonads and xanthomonads. *Physiol. Plant Pathol.* 15:341-349.
  12. Fenselau, S., Balbo, I., and Bonas, U. 1992. Determinants of pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. *Mol. Plant-Microbe Interact.* 5:390-396.
  13. Fett, W. F., and Dunn, M. F. 1989. Exopolysaccharides produced by phytopathogenic *Pseudomonas syringae* pathovars in infected leaves of susceptible hosts. *Plant Physiol.* 89:5-9.
  14. Flor, A. H. 1947. Host-parasite interactions in flax rust - its genetics and other implications. *Phytopathology* 45:680-685.
  15. Genin, S., Gough, C. L., Zischek, C., and Boucher, C. A. 1992. Evidence that the *hrpB* gene encodes a positive regulator of pathogenicity genes from *Pseudomonas solanacearum*. *Mol. Microbiol.* 6:3065-3076.
  16. Goodman, R. N., and Novacky, A. 1994. The hypersensitive reaction in plants to pathogens: A resistance phenomenon. *American Phytopathological Society*, St. Paul, MN.
  17. Gough, C. L., Genin, S., Zischek, C., and Boucher, C. A. 1992. *hrp* genes of *Pseudomonas solanacearum* are homologous to pathogenicity determinants of animal pathogenic bacteria and are conserved among plant pathogenic bacteria. *Mol. Plant-Microbe Interact.* 5:384-389.
  18. Grimm, C., and Panopoulos, N. J. 1989. The predicted protein product of a pathogenicity locus from *Pseudomonas syringae* pv. *phaseolicola* is homologous to a highly conserved domain of several prokaryotic regulatory proteins. *J. Bacteriol.* 171:5031-5038.
  19. Gross, D. C. 1991. Molecular and genetic analysis of toxin production by pathovars of *Pseudomonas syringae*. *Annu. Rev. Phytopathol.* 29:247-278.
  20. He, S. Y., Huang, H.-C., and Collmer, A. 1993. *Pseudomonas syringae* pv. *syringae* harpin<sub>ps</sub>: A protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. *Cell* 73:1255-1266.
  21. He, S. Y., Lindeberg, M. L., and Collmer, A. 1993. Protein secretion by plant pathogenic bacteria. Pages 39-64 in: *Biotechnological Prospects for Plant Pathogen Control*. I. Chet, ed. John Wiley & Sons, New York.
  22. Huang, H.-C., He, S. Y., Bauer, D. W., and Collmer, A. 1992. The *Pseudomonas syringae* pv. *syringae* 61 *hrpH* product: An envelope protein required for elicitation of the hypersensitive response in plants. *J. Bacteriol.* 174:6678-6885.
  23. Huang, H.-C., Lin, R.-H., Chang, C.-J., Collmer, A., and Deng, W.-L. 1995. The complete *hrp* gene cluster of *Pseudomonas syringae* pv. *syringae* 61 includes two blocks of genes required for harpin<sub>ps</sub> secretion that are arranged collinearly with *Yersinia* *ycs* homologs. *Mol. Plant-Microbe Interact.* 8:733-746.
  24. Huang, H. C., Schuurink, R., Denny, T. P., Atkinson, M. M., Baker, C. J., Yucel, I., Hutcheson, S. W., and Collmer, A. 1988. Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco. *J. Bacteriol.* 170:4748-4756.
  25. Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. 1989. Bacterial blight of soybean: Regulation of a pathogen gene determining host cultivar specificity. *Science* 245:1374-1377.
  26. Innes, R. W., Bent, A. F., Kunkel, B. N., Bisgrove, S. R., and Staskawicz, B. J. 1993. Molecular analysis of avirulence gene *avrBs2* and identification of a putative regulatory sequence common to all known *Pseudomonas syringae* avirulence genes. *J. Bacteriol.* 175:4859-4869.
  27. Keen, N. T. 1990. Gene-for-gene complementarity in plant-pathogen interactions. *Annu. Rev. Genet.* 24:447-463.
  28. Keen, N. T., Tamaki, S., Kobayashi, D., Gerhold, D., Stayton, M., Shen, H., Gold, S., Lorang, J., Thordal-Christensen, H., Dahlbeck, D., and Staskawicz, B. 1990. Bacteria expressing avirulence gene D produce a specific elicitor of the soybean hypersensitive reaction. *Mol. Plant-Microbe Interact.* 3:112-121.
  29. Klement, Z. 1963. Rapid detection of pathogenicity of phytopathogenic pseudomonads. *Nature* 199:299-300.
  30. Knoop, V., Staskawicz, B., and Bonas, U. 1991. Expression of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria* is not under the control of *hrp* genes and is independent of plant factors. *J. Bacteriol.* 173:7142-7150.
  31. Laby, R. J., and Beer, S. V. 1992. Hybridiza-



Suresh Gopalan

Dr. Gopalan is a research associate at the Department of Energy Plant Research Laboratory, Michigan State University. He received his B.S. degree in mechanical engineering and his M.S. degree in bioscience under the dual degree program at the Birla Institute of Technology and Science, India, in 1983. He received his Ph.D. degree in biotechnology at the Center for Biotechnology, Anna University, India, in 1994. His thesis was on the development of a biopesticide based on *Bacillus sphaericus* for mosquito control. He joined S. Y. He's laboratory in October 1993 as a postdoctoral fellow at the University of Kentucky. In 1995, he moved with He to the DOE Plant Research Laboratory. His current research focuses on molecular aspects of plant responses to bacterial pathogens.



S. Y. He

Dr. He is an assistant professor in the Department of Energy Plant Research Laboratory at Michigan State University. He received his B.S. and M.S. degrees in plant protection at Zhejiang Agricultural University, People's Republic of China, and his Ph.D. degree in plant pathology at Cornell University. He joined the faculty of the University of Kentucky in 1993. In 1995, he moved to Michigan State University. His research interests are in molecular plant-microbe interactions.



- tion and functional complementation of the *hrp* gene cluster from *Erwinia amylovora* strain Ea321 with DNA of other bacteria. *Mol. Plant-Microbe Interact.* 5:412-419.
32. Lamb, C. J., Lawton, M. A., Dron, M., and Dixon, R. A. 1989. Signals and transduction mechanisms for activation of plant defenses against microbial attack. *Cell* 56:215-224.
  33. Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. 1986. Gene cluster of *Pseudomonas syringae* pv. *phaseolicola* controls pathogenicity of bean plants and hypersensitivity on nonhost plants. *J. Bacteriol.* 168:512-522.
  34. Marda, G. B., Brommonschenkel, S. H., Chumwongse, J., Frary, A., Ganai, M. W., Spivey, R., Wu, T., Earle, E. D., and Tanksley, S. D. 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science* 262:1432-1436.
  35. Nisepold, F., Anderson, D., and Mills, D. 1985. Cloning determinants of pathogenesis from *Pseudomonas syringae* pathovar *syringae*. *Proc. Natl. Acad. Sci. USA* 82:406-410.
  36. Preston, G., Huang, H.-C., He, S. Y., and Collmer, A. 1995. The *HrpZ* proteins of *Pseudomonas syringae* pvs. *syringae*, *glycinis*, and *tomato* are encoded by an operon containing *Yersinia* ysc homologs and elicit the hypersensitive response in tomato but not in soybean. *Mol. Plant-Microbe Interact.* 8:717-732.
  37. Rahme, L. G., Mindrinos, M. N., and Panopoulos, N. J. 1992. Plant and environmental sensory signals control the expression of *hrp* genes in *Pseudomonas syringae* pv. *phaseolicola*. *J. Bacteriol.* 174:3499-3507.
  38. Salmeron, J. M., and Staskiewicz, B. J. 1993. Molecular characterization and *hrp* dependence of the avirulence gene *avrPto* from *Pseudomonas syringae* pv. *tomato*. *Mol. Gen. Genet.* 239:6-16.
  39. Salmond, G. C. 1994. Secretion of extracellular virulence factors by plant pathogenic bacteria. *Annu. Rev. Phytopathol.* 32:181-200.
  40. Shen, H., and Keen, N. T. 1993. Characterization of the promoter of avirulence gene D from *Pseudomonas syringae* pv. *tomato*. *J. Bacteriol.* 175:5916-5924.
  41. Sholte, R., and Bonas, U. 1992. A *Xanthomonas* pathogenicity locus is induced by sucrose and sulfur-containing amino acids. *Plant Cell* 4:79-86.
  42. Silverstone, S. E., Gilchrist, D. G., Bostock, R. M., and Kosuga, T. 1993. The 73-kb pLAA plasmid increases competitive fitness of *Pseudomonas syringae* subspecies *savastanoi* in oleander. *Curr. Plant Sci. Biotechnol. Agric.* 14:659-664.
  43. Staskiewicz, B. J., Ausubel, F. M., Baker, B. J., Ellis, J. G., and Jones, J. D. G. 1995. Molecular genetics of plant disease resistance. *Science* 268:661-667.
  44. Staskiewicz, B. J., Dahlbeck, D., and Keen, N. T. 1984. Cloned avirulence gene of *Pseudomonas syringae* pv. *glycinis* determines race-specific incompatibility on *Glycine max* (L.) Merr. *Proc. Natl. Acad. Sci. USA* 81:6024-6028.
  45. Van Gijsegem, F., Genin, S., and Boucher, C. 1995. *hrp* and *avr* genes, key determinants controlling the interactions between plants and Gram-negative phytopathogenic bacteria. Pages 273-292 in: *Pathogenesis and Host Specificity in Plant Diseases: Biochemical, Biochemical, Genetic and Molecular Bases*. Vol. 1, Prokaryotes. U. S. Singh, R. P. Singh, and K. Kohmoto, eds. Pergamon, U.K.
  46. Van Gijsegem, F., Gough, C., Zischak, C., Niqueux, E., Arlat, M., Genin, S., Barberis, P., German, S., Castello, P., and Boucher, C. 1995. The *hrp* gene locus of *Pseudomonas solanacearum*, which controls the production of a type III secretion system, encodes eight proteins related to components of the bacterial flagellar biogenesis complex. *Mol. Microbiol.* 15:1095-1114.
  47. Wei, Z.-M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A., and Beer, S. V. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* 257:85-88.
  48. Wei, Z.-M., Sneath, B. J., and Beer, S. V. 1992. Expression of *Erwinia amylovora* *hrp* genes in response to environmental stimuli. *J. Bacteriol.* 174:1875-1882.
  49. Willis, D. K., Barta, T. M., and Kinscherf, T. G. 1991. Genetics of toxin production and resistance in phytopathogenic bacteria. *Experimentia* 47:765-771.
  50. Willis, D. K., Rich, J. J., and Hrabak, E. M. 1991. *hrp* genes of phytopathogenic bacteria. *Mol. Plant-Microbe Interact.* 4:132-138.
  51. Xiao, Y., Heu, S., Li, J., Lu, Y., and Hutcheson, S. W. 1994. Identification of a putative alternative sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* and *ArmA* genes. *J. Bacteriol.* 176:1025-1036.
  52. Xiao, Y., Lu, Y., Heu, S., and Hutcheson, S. W. 1992. Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 *hrp* cluster. *J. Bacteriol.* 174:1734-1741.
  53. Yucel, I., Xiao, Y., and Hutcheson, S. V. 1989. Influence of *Pseudomonas syringae* culture conditions on initiation of the hypersensitive response of cultured tobacco cells. *Appl. Environ. Microbiol.* 55:1724-1729.

**NEW BOOKS**

## Healthy Plants Start Here!

Order 3 or more books, SAVE 10%!

**APS PRESS**



### Compendium of Cucurbit Diseases

Edited by  
Thomas A. Zitter,  
Donald L. Hopkins, and  
Claude E. Thomas

U.S. \$35; Elsewhere \$44  
ISBN 0-89054-207-4 (Item No. 42074)



### Compendium of Flowering Potted Plant Diseases

By  
Margery L. Daughtrey,  
Robert L. Wick, and  
Joseph L. Peterson

U.S. \$35; Elsewhere \$44  
ISBN 0-89054-202-3 (Item No. 42023)



### Compendium of Stone Fruit Diseases

Edited by J.M. Ogawa,  
E.I. Zehr,  
G.W. Bird, D.F. Ritchie,  
K. Uria, and  
J.K. Uemoto

U.S. \$35; Elsewhere \$44  
ISBN 0-89054-174-4 (Item No. 41744)

Title	Item No.	Qty.	Price	Subtotal
Cucurbit Diseases	42074			
Flowering Potted Plant Diseases	42023			
Stone Fruit Diseases	41744			

Book Subtotal

3-Book Discount

If you are ordering three or more books, enter the amount from the line above minus 10%.

**ORDER TOLL FREE**  
**1-800-328-7560**  
In the U.S. and most of Canada,  
8 a.m.-5 p.m. CST.  
Please have your credit  
card ready.  
Elsewhere: 612/434-7250  
FAX: 612/434-0768

Current, **PAID** members of **APS ONLY**  
Enter amount from last line completed  
minus your discount of 15% (U.S.) or 25% (Elsewhere).  
Minnesota/Canadian Residents  
Enter amount from last line completed plus 6.5% sales tax (MVA)  
or 7% GST tax (Canada) GST # R128886123.  
Shipping & Handling  
U.S. book orders add \$3.50. Elsewhere, see information below.  
Total  
Enter amount from last line completed.

Mail to: APS PRESS  
The American Phytopathological Society  
1340 Plover Road, St. Paul, MN 55121-2097 U.S.A.

Make checks payable to APS in U.S. funds through a member bank of the U.S. Federal Reserve System. Allow 2 to 3 weeks for delivery in the U.S. Elsewhere, please include standard surface postage for delivery in 4-6 weeks; expedited shipping available upon request at an additional charge for delivery in 2-3 weeks.

- ☐ Payment enclosed is \_\_\_\_\_  
☐ Unavailable in the USA may be billed. You must include a written purchase order, contact name, and phone number.  
☐ Charge my: ☐ VISA (13 or 16 digits) ☐ American Express (15 digits)  
☐ MasterCard (16 digits) ☐ Diners (14 digits)

413K

Card No.

Exp. Date

Name

Co./Inst.

Address

City/State

Zip/Country

Phone (include if shipping to a P.O. box)

Fax

#M7096-4/96



# Bacterial home goal by harpins

Ulla Bonas

**H**ost-pathogen interactions are dynamic and multifactorial; whether a microorganism succeeds or fails in colonizing a potential host depends on factors from both organisms. A successful pathogen has to overcome the defenses of the host. In bacteria that are pathogenic for animals or for plants, particularly Gram-negative organisms, a large number of genes are essential to infect host tissue and establish disease. Expression of these genes is generally controlled by environmental conditions such as temperature, pH, salt concentration and nutrient availability<sup>1,2</sup>.

## Pathogenicity, hypersensitive reaction and elicitors

In the Gram-negative plant pathogens *Erwinia*, *Pseudomonas* and *Xanthomonas*, genes organized in clusters of 25–40 kb are fundamentally involved in any obvious interaction with a plant (for a review see Ref. 3). These genes have been designated *hrp* (hypersensitive reaction and pathogenicity) because they are essential not only for pathogenicity towards a susceptible host plant, but also for interaction with resistant host varieties and with plants that are not a host for that pathogen. In plants, the hypersensitive reaction (HR) (Ref. 4) is a rapid defense reaction involving localized plant cell death and production of substances such as phenolics and phytoalexins at the site of infection. The HR prevents pathogen spread and thus halts disease development.

In the wild, plants are resistant to the majority of pathogens. The HR, therefore, is an important defense mechanism against all kinds of possible disease agents (bacteria, fungi, nematodes and viruses). It is not only important to interactions of pathogens with nonhost plants, but also to interactions between plants that carry resistance genes and microorganisms that are pathogens for that species.

Although the genes involved in plant defense<sup>5,6</sup> are becoming better understood, very little is known about the nature of the initial signals and their perception. Induction of the HR in a bacterium-plant interaction requires functional *hrp* genes and appears to be mediated by signal molecules or 'elicitors'. Recent DNA sequence analyses indicate that several putative Hrp proteins from different species are related and may be involved in a secretion system reminiscent of secretion of Yops (*Yersinia* outer proteins) in *Yersinia*<sup>7–11</sup>. So far, only one specific elicitor of the HR in a bacterium-plant interaction has been described. The *avrD* gene from *Pseudomonas syringae* pv. *tomato* mediates production of a low-molecular-mass compound that specifically induces the HR only in the soybean plant (a nonhost) when it carries the corresponding *Rpg4* resistance gene<sup>5,12</sup>.

## Harpins

Recently, two bacterial HR-inducing proteins, called 'harpins', were identified in *Erwinia amylovora*<sup>13</sup> and *P. syringae* pv. *syringae*<sup>14</sup>. Although the harpins differ in primary sequence, they have several features in common: they are glycine rich and heat stable, and they both induce an HR in tobacco, a nonhost plant for these bacteria. The genes encoding harpins are localized within the *hrp* clusters and obviously have a dual role in that they are also required for pathogenicity towards the normal host plant. Both *hrp* clusters allow nonpathogenic bacteria, such as *Escherichia coli*, to induce an HR in tobacco after recombinant expression, suggesting that the genes for the tobacco HR elicitors are present within the clusters<sup>15,16</sup>.

The first harpin to be identified, harpin<sub>Er</sub>, is a cell-envelope-associated protein encoded by the *hrpN* gene of *Er. amylovora*, a pathogen of pear and apple<sup>13</sup>. Recently, He and co-workers<sup>14</sup> have used an elegant approach to identify harpin<sub>Ps</sub>, which is encoded by the *hrpZ* gene in the bean pathogen *P. s. pv. syringae*. Lysates of an expression library in *E. coli*, made using the cloned *P. s. pv. syringae* *hrp* cluster, were directly screened for HR-inducing activity on tobacco leaves. Two proteins were identified, one of which was an amino-terminal deletion of harpin<sub>Ps</sub> with even higher activity than the full-size protein; whether processing occurs during natural infection is not clear. Interestingly, the carboxyl terminus contains two short, direct repeats that are essential for elicitor activity. The activity is in the same range as that of the *Erwinia* harpin<sub>Er</sub>; however, to elicit an HR in other plants requires higher levels of the elicitor. He *et al.* show convincingly that the secretion of harpin<sub>Ps</sub> by *P. s. pv. syringae* depends on a product called HrpH that is closely related to proteins in other plant pathogens, and also in animal pathogens such as *Yersinia* and *Shigella*, where they are essential for protein secretion<sup>9,10,14</sup>.

These exciting findings help verify the model that Hrp proteins are involved in the transport of elicitors and virulence factors<sup>7</sup>. Not surprisingly, the results presented by He and co-workers<sup>14</sup> also stimulate many questions. It needs to be shown that harpin<sub>Ps</sub> is actually secreted when the bacterium interacts with tobacco tissue (the *hrp* genes were induced *in vitro*). The concentration needed for HR induction (more than 600 nM) is much higher than one would expect for specific signal molecules. Are harpins toxins? Most importantly, what is their function in pathogenicity, and why do they

U. Bonas is in the CNRS-Institut des Sciences Végétales, Avenue de la Terrasse, F-91198 Gif-sur-Yvette, France.

not elicit an HR in the host plant? Are harpins the only elicitors of nonhost HR in tobacco and possibly in other plants? Is the same mechanism used in tobacco to recognize both the *Erwinia* and the *P. s. pv. syringae* harpins? Is host resistance different in mechanism from nonhost resistance? Answers to this fascinating puzzle require the identification of more HR elicitors and their putative plant receptors.

#### References

- 1 Mekalanos, J.J. (1992) *J. Bacteriol.* 174, 1-7
- 2 Long, S.R. and Staskawicz, B. (1993) *Cell* 73, 921-935
- 3 Willis, D.K., Rich J.J. and Hrabak, E.M. (1991) *Mol. Plant-Microbe Interact.* 4, 132-138
- 4 Klement, Z. (1982) in *Phytopathogenic Prokaryotes* (Vol. 2) (Mount, M.S. and Lacy, G.H., eds), pp. 149-177, Academic Press
- 5 Keen, N.T. (1992) *Plant Mol. Biol.* 19, 109-122
- 6 Lindsay, W.P., Lamb, C.J. and Dixon, R.A. (1993) *Trends Microbiol.* 1, 181-187
- 7 Fenselau, S., Balbo, I. and Bonas, U. (1992) *Mol. Plant-Microbe Interact.* 5, 390-396
- 8 Gough, C.L. et al. (1992) *Mol. Plant-Microbe Interact.* 5, 384-389
- 9 Huang, H-C. et al. (1992) *J. Bacteriol.* 174, 6878-6885
- 10 Van Gijsegem, F., Genin, S. and Boucher, C. (1993) *Trends Microbiol.* 1, 175-180
- 11 Huang, H-C. et al. (1993) *Mol. Plant-Microbe Interact.* 6, 515-520
- 12 Smith, M.J. et al. (1993) *Tetrahedron Lett.* 34, 223-226
- 13 Wei, Z-M. et al. (1992) *Science* 257, 85-88
- 14 He, S.Y., Huang, H-C. and Collmer, A. (1993) *Cell* 73, 1255-1266
- 15 Huang, H-C. et al. (1988) *J. Bacteriol.* 170, 4748-4756
- 16 Beer, S.V. et al. (1991) in *Advances in Molecular Genetics of Plant-Microbe Interactions* (Hennecke, H. and Verma, D.P.S., eds), pp. 53-60, Kluwer Academic Publishers

# Initiation and spread of $\alpha$ -herpesvirus infections

Thomas C. Mettenleiter

**H**erpesviruses are large animal viruses with a DNA genome varying from approximately 120 to 250 kb. Based on their biological properties, the Herpesviridae have been divided into three subfamilies, the  $\alpha$ -,  $\beta$ - and  $\gamma$ -herpesvirinae, prototypes of which are the human pathogens herpes simplex virus (HSV), cytomegalovirus (HCMV) and Epstein-Barr virus (EBV), respectively. As enveloped viruses, they depend on two consecutive processes for infectious entry into target cells: (1) attachment of free virions to cells and (2) penetration, that is, fusion of virion envelope and cellular cytoplasmic membrane leading to release of the nucleocapsid into the cell. Virion envelope glycoproteins play important roles in both these processes (see Refs 1,2 for recent reviews).

After infection of primary target cells, virus spread can occur by several different mechanisms. Infected cells may release infectious

virions that reinitiate infection from outside. In addition, direct viral cell-to-cell spread from primary infected cells to adjacent non-infected cells may occur. In the host, virus may be disseminated by circulating infected cells that adhere to noninfected tissues and transmit infectivity directly. Recent results on HSV and pseudorabies virus (PrV) shed more light on these processes in  $\alpha$ -herpesviruses. PrV causes Aujeszky's disease in swine, which is characterized by nervous and respiratory symptoms, and reproductive failure. Unlike HSV, PrV is not pathogenic for humans. However, the two viruses have several features in common, including a broad host range *in vitro*, and several species besides the natural host can be infected experimentally. In addition, all of the known PrV glycoproteins are

related to homologous glycoproteins in HSV (Ref. 1)\*.

#### Attachment

Binding of free infectious virus to target cells involves interactions between virion envelope glycoproteins and cellular virus receptors. Herpes virions contain a large number of different virus-encoded envelope glycoproteins that might participate in attachment. A well-known example of a cellular herpesvirus receptor is the B-cell membrane protein CR2 (CD21), which binds EBV (Ref. 3). Recent studies have demonstrated that several  $\alpha$ - (reviewed in Ref. 1),  $\beta$ - and  $\gamma$ -herpesviruses<sup>4,5</sup> bind to their target cells by interaction of virion components with cell-surface glycosaminoglycans, principally heparan sulfate (HS)<sup>6</sup>.

T.C. Mettenleiter is in the Federal Research Centre for Virus Diseases of Animals, PO Box 1149, D-72001 Tübingen, Germany.

\*At the 18th International Herpesvirus Workshop, a common nomenclature for  $\alpha$ -herpesvirus glycoproteins was agreed on, based on designations of HSV glycoproteins. This nomenclature is used here.

- Turner JE, Novacky A (1974) The quantitative relation between plant and bacterial cells involved in the hypersensitive reaction. *Phytopathology* 64: 885-90
- Van Gijsegem F (1986) Analysis of the pectin-degrading enzymes secreted by three strains of *Erwinia chrysanthemi*. *J Gen Microbiol* 132: 617-624
- Van Gijsegem F (1989) Relationship between the pel genes of the pelADE cluster in *Erwinia chrysanthemi* strain B374. *Mol Microbiol* 3: 1415-1424
- Van Gijsegem F, Gemin S, Boucher C (1993) Evolutionary conservation of pathogenicity determinants among plant and animal pathogenic bacteria. *Trends Microbiol* 1: 175-180
- Vayda ME, Schaeffer HJ (1988) Hypoxic stress inhibits the appearance of wound-response proteins in potato tubers. *Plant Physiol* 88: 805-809
- Vayda ME, Antonov LS, Yang Z, Butler WD, Lacey GH (1992) Hypoxic stress inhibits aerobic wound-induced resistance and activates hypoxic resistance to bacterial soft rot. *Am Potato J* 69: 239-253
- Wanderer C (1989) Secretion, processing and activation of bacterial extracellular proteases. *Mol Microbiol* 3: 1825-1831
- Wanderer C, Delapierre P, Letoffe S, Schwartz M (1997) Characterization of *Erwinia chrysanthemi* extracellular proteases: cloning and expression of the protease genes in *Escherichia coli*. *J Bacteriol* 169: 5046-5053
- Wei Z-M, Laby RJ, Zurell CH, Bauer DW, Ito SY, Collmer A, Beer SV (1992) Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* 257: 85-88
- Wien MC, Inness RW, Bent AF, Slaskawicz BJ (1991) Identification of *Pseudomonas syringae* pathogens of Arabidopsis and a bacterial locus determining avirulence on both Arabidopsis and soybean. *Plant Cell* 3: 49-59
- Willits DK, Baria TM, Kirschner J (1991a) Genetics of toxin production and resistance in phytopathogenic bacteria. *Experientia* 47: 765-771
- Willits DK, Rich JJ, Hrabak EM (1991b) hrp genes of phytopathogenic bacteria. *Mol Plant Microbe Interact* 4: 132-138
- Xian Y, Lu Y, Hsu S, Hutcheson SW (1992) Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 hrp cluster. *J Bacteriol* 174: 1734-1741
- Yamazaki N, Fry SC, Darvill AG, Albersheim P (1983) Host-pathogen interactions. XXIV. Fragments isolated from suspension-cultured sycamore cell walls inhibit the ability of the cells to incorporate [<sup>14</sup>C] leucine into proteins. *Plant Physiol* 72: 884-889
- Yang Z, Clamer CL, Lacey GH (1992) *Erwinia carotovora* subsp. *carotovora* pectic enzymes: In planta gene activation and roles in soft-rot pathogenesis. *Mol Plant Microbe Interact* 5: 104-112
- Yoder MD, Keen NT, Jurak F (1993) New domain motif: the structure of pectate lyase C, a secreted plant virulence factor. *Science* 260: 1503-1507
- Young JM (1974) Development of bacterial populations in vivo in relation to plant pathogenicity. *NZ J Agric Res* 17: 105-113
- Yucel I, Xiao Y, Hutcheson SW (1989) Influence of *Pseudomonas syringae* culture conditions on initiation of the hypersensitive response of cultured tobacco cells. *Appl Environ Microbiol* 55: 1724-1729
- Zink RJ, Engvall JK, McEvoy JJ, Chatterjee AK (1985) *reca* is required in the induction of pectin lyase and carotovorin in *Erwinia carotovora* subsp. *carotovora*. *J Bacteriol* 164: 390-396
- Zier M, Hankin L (1970) Regulation of pectate lyase synthesis in *Pseudomonas fluorescens* and *Erwinia carotovora*. *J Bacteriol* 104: 13-18
- Zucker M, Hankin L, Sands D (1972) Factors governing pectate lyase synthesis in soft rot and non-soft rot bacteria. *Physiol Plant Pathol* 2: 59-67

## hrp Genes of Phytopathogenic Bacteria

U. BONAS

Bonas, U. 1994. *hrp* genes of phytopathogenic bacteria. Pages 79-98 in: *Current Topics in Microbiology and Immunology*, Vol. 192: *Bacterial Pathogenesis of Plants and Animals - Molecular and Cellular Mechanisms*. J. L. Dangl, ed. Springer-Verlag, Berlin.

1	Introduction	79
2	Isolation of <i>hrp</i> Genes and General Features	80
3	Structural Organisation and Relatedness of <i>hrp</i> Clusters	83
4	Function of <i>hrp</i> Genes in <i>Xanthomonas campestris</i> pv. <i>vesicatoria</i> and Other Plant Pathogenic Bacteria	83
5	<i>hrp</i> -dependent Secretion of Hypersensitive Response-Inducing Proteins	89
5.1	Harpin from <i>Erwinia amylovora</i>	89
5.2	Harpin from <i>Pseudomonas syringae</i> pv. <i>syringae</i>	89
5.3	PopA from <i>Pseudomonas solanacearum</i>	90
6	Regulation of Expression of <i>hrp</i> Genes	90
6.1	<i>Pseudomonas syringae</i>	91
6.2	Regulatory Genes <i>hrpH5</i> and <i>rpod</i> of <i>Pseudomonas syringae</i> pv. <i>phaseolicola</i>	91
6.3	Conserved Sequence Boxes in <i>Pseudomonas syringae</i>	92
6.4	<i>Xanthomonas campestris</i>	93
6.5	<i>Erwinia</i> and <i>Pseudomonas solanacearum</i>	93
	References	94

## 1 Introduction

In nature plants are resistant to the majority of pathogens, and many bacteria live in close contact with the plant without causing any harm (see chapter by Beatrice and Lindow in this volume). Among the 1600 different species known in the bacterial kingdom only a small number (about 80) are plant pathogenic and in most cases highly specialized with respect to the plant that can be attacked. Only a few of these species are gram-positive, e.g., *Clavibacter* spp. and *Streptomyces* spp. In this review I focus on subspecies of the gram-negative genera *Erwinia*, *Pseudomonas*, and *Xanthomonas*, which comprise the major bacterial plant pathogens.

To be a successful pathogen the invading bacterium has to overcome the plant's defense. During evolution plant pathogenic bacteria have acquired multiple functions that enable them to colonize and multiply in living plant tissue. In nature, bacteria enter the plant through natural openings (stomata, hydathodes) or

wounds. The bacterial armory contains a number of weapons that contribute to pathogenicity. Obvious examples include degradative extracellular enzymes such as pectinases, cellulases, and proteases. When the corresponding genes are mutated, bacterial ability to invade plant tissues is more or less affected depending on the pathogen, i.e., these functions contribute to and modulate development and severity of infection to different extents (see chapters by Dow and Daniels, and Colmer and Bauer in this volume).

In addition, phytopathogenic bacteria possess a large number of genes needed for basic pathogenicity. These genes have been operationally defined as *hrp* (hypersensitive reaction and pathogenicity; LINDGREN et al. 1986) based on their mutant phenotype. *hrp* genes are not only essential for pathogenicity on a plant, i.e., the ability to cause disease in a compatible interaction, but also for the incompatible interaction with resistant host varieties or with plants that are not normally a host for the particular pathogen (so called non-host). The incompatible interaction is often associated with the induction of a hypersensitive reaction (HR) in the plant. In contrast to the use of the term hypersensitivity in the animal field, in plants the HR is a rapid defense response involving localized plant cell death, production of phenolics and antimicrobial agents, e.g., phytoalexins, at the site of infection (KLEMEYER 1982; LINDSAY et al. 1993). The HR results in prevention of pathogen multiplication and spread and thus in prevention of disease development. Under natural infection conditions the HR is microscopically small and can be induced by just one bacterial cell. Only when bacteria are introduced into plant tissue at high cell densities in the laboratory (about  $10^7$  colony forming units or more/ml) is the HR macroscopically visible as confluent necrosis and can be clearly distinguished from typical disease symptoms. It is important to note that saprophytic or nonpathogenic bacteria such as *Escherichia coli* or *Pseudomonas fluorescens* do not induce the HR and are unable to multiply in plant tissue.

## 2 Isolation of *hrp* Genes and General Features

*hrp* genes have been isolated from all major gram-negative plant pathogenic bacteria except *Agrobacterium*. There are excellent reviews that describe the early work or focus more on one particular pathogen (WILKUS et al. 1991; BOUCHIER et al. 1992). The majority of *hrp* genes have been identified by complementation of loss-of-function mutants. Mutants obtained by random chemical (e.g., N-methyl-N-nitro-N-nitrosoguanidine) or transposon mutagenesis of a pathogenic wild-type strain were inoculated into the host plant and screened for loss of both the ability to cause disease in susceptible plants and to induce the HR in resistant host or non-host plants (often tobacco). The second criterion for the isolation of genes specific for the plant interaction was to ensure that the mutants would still grow in minimal medium. This way mutants affected in genes for basic housekeeping functions were eliminated. A third characteristic of all *hrp* mutants is that they are unable to grow in the plant.

The *hrp* genes were originally described for the bean pathogen *Pseudomonas syringae* pv. *phaseolicola*. LINDGREN and coworkers (1986) isolated Tr5-induced mutants of *P. s. pv. phaseolicola* that had lost both the ability to induce halo-blight disease on bean and the HR in tobacco. Complementation with cosmid clones from a genomic library of the wild-type strain resulted in isolation of a cluster of *hrp* genes localized in a 20 kb DNA region. This was the first indication that both the ability to cause disease and to induce the HR are mediated by common steps in a "pathway".

Since then *hrp* gene clusters have been cloned from a number of different bacteria. Examples include *Pseudomonas solanacearum* (BOUCHIER et al. 1987; Fig. 1B), the *Xanthomonas campestris* pathogens *campestris* and *vitis* (AARAI et al. 1991), *translucens* (WANEY et al. 1991), and *vesicatoria* (BOWAS et al. 1991; Fig. 1A), *Erwinia amylovora* (STEINBERGER and BEER 1988; BARNY et al. 1990; WALTERS et al. 1990; BAUER and BEER 1991), and several other pathogens of *P. syringae* (e.g., HUANG et al. 1988; LINDGREN et al. 1988; Fig. 1C). In addition, genes with DNA homology, and in some cases functional homology, have been isolated from other species, e.g., the so-called *wts* genes from *E. stewartii* (CORLIN et al. 1992; LAY and BEER 1992), and a region containing pathogenicity genes from *X. c. pv. glycines* that complement *hrp* mutants of *X. c. pv. vesicatoria* (HUANG et al. 1992; BOWAS, unpublished results). Interestingly, nonpathogenic xanthomonads that were originally isolated from diseased plants as opportunists together with pathogenic bacteria do not contain *hrp*-related DNA sequences (STRAL and MINSWAGE 1990; BOWAS et al. 1991). In *Agrobacterium tumefaciens* or in strains of *Rhizobium* spp. there seem to be no *hrp* gene equivalents present (BOWAS et al. 1991; LAY and BEER 1992). This conclusion is based on DNA hybridization experiments and, of course, does not exclude the presence of genes with functional homology to *hrp* genes in these species.

In all of the cases mentioned above, the *hrp* genes are organized in clusters of 22–40 kb, and I will restrict most of this chapter to these large *hrp* clusters. In addition, several smaller *hrp* loci have been described that are not linked to the large cluster present in the same bacterium. These include a region in *P. solanacearum* (HUANG et al. 1990), the *hrpX* locus that is conserved in *X. campestris* pathogens *campestris* (KAMOUN and KADO 1990; KAMOUN et al. 1992) and *oryzae* (KAMOUN et al. 1993), and the *hrpM* locus in *P. s. pv. syringae* (NIEROLD et al. 1985; MUKHOPADHYAY et al. 1988). *hrpM* is functionally conserved in pathovar *phaseolicola* (FELLAY et al. 1991). Besides being nonpathogenic and unable to induce the HR in tobacco, *P. syringae hrpM* mutants are also affected in mucus production. The *hrpM* locus encodes two putative proteins which are similar and have been shown to be functionally homologous to the products of the *E. coli mdoGH* operon (LOUBENS et al. 1993). The *mdoGH* genes are required for periplasmic membrane-derived oligosaccharide synthesis in *E. coli*. The *hrpQ* and *hrpJ* genes from *P. s. pv. phaseolicola* (MILLEN et al. 1993) will be discussed later in this chapter.

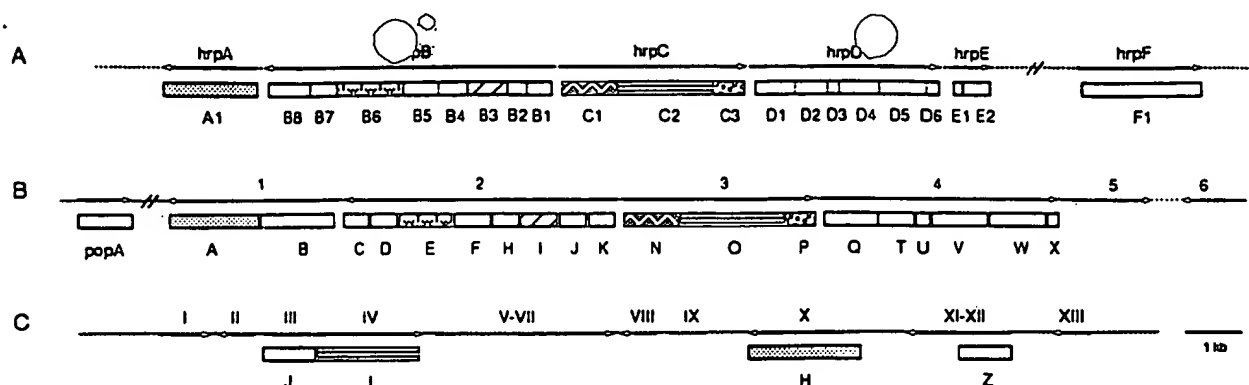


Fig. 1A-C. Genetic and translational organization of the *hrp* gene cluster of different plant pathogenic bacteria. A *Xanthomonas campestris* pv. *vesicatoria*; B *Pseudomonas solanacearum*; and C *Pseudomonas syringae* pv. *syringae*. Arrows represent transcription units as determined by genetic analyses. Boxes correspond to sequences of open reading frames (ORFs) that have been published. In case of sequence similarities between ORFs in different clusters the boxes are filled with the same pattern. For references, see text

### 3 Structural Organization and Relatedness of *hrp* Clusters

Genetic studies using transposon-induced insertion mutants in the respective bacterial wild-type strains revealed that the *hrp* clusters contain at least six to eight complementation groups (Fig. 1). Some *hrp* gene clusters have clearly been shown to be localized in the chromosome, e.g., in *P.s. pv. phaseolicola* (Rahme et al. 1991) and in *X.c. pv. vesicatoria* (Bonas et al. 1991), whereas in *P. solanacearum*, the *hrp* cluster is on a megaplasmid (Boucher et al. 1987).

Striking similarities have recently been found between the *hrp* genes of pathogens belonging to different genera. The first indication of homologies came from Southern hybridization studies. DNA homology was observed among different strains of the same pathovar, as well as between pathovars or strains within a species, and in some cases also between species. However, the degree of conservation varies. DNA homology is high within pathovars of a given subspecies, e.g., in *P. syringae* (Lindgren et al. 1988; Huang et al. 1991) and in *X. campestris* (Bonas et al. 1991). The latter studies were recently extended by PCR using primers based on *hrp* sequences from *X.c. pv. vesicatoria* (Lere et al. 1994). Furthermore, at least some regions of the *hrp* clusters appear to be conserved on the DNA level between *P. solanacearum* and pathovars of *X. campestris*, *P. syringae*, and also to *E. amylovora* (Boucher et al. 1987; Arlat et al. 1991; Gough et al. 1992; Laevy and Beer 1992). In addition, cross-complementation within a subspecies indicated a high degree of functional conservation of *hrp* genes (e.g., Lindgren et al. 1988; Arlat et al. 1991; Bonas et al. 1991; Laevy and Beer 1992). Due to sequence data it is now becoming more and more apparent that several *hrp* genes are conserved in all major gram-negative plant pathogenic bacteria (see below). Whether there are *hrp* genes that are clearly pathovar-specific can only be answered when complete sequence information becomes available for several *hrp* clusters.

### 4 Function of *hrp* Genes in *Xanthomonas campestris* pv. *vesicatoria* and Other Plant Pathogenic Bacteria

DNA sequence analysis of the *hrp* genes has revealed some important clues to their possible biochemical functions. One of the first genes sequenced was a regulatory gene, *hrpS*, from *P.s. pv. phaseolicola* (Grimm and Panopoulos 1989). This gene as well as *hrpB*, a regulatory gene from *P. solanacearum* (Genin et al. 1992), will be discussed below in the context of gene regulation.

Since *hrp* genes are environmentally regulated (see below), it was believed for a while that they would be encoding "alternative" proteins required for adaptation of the bacterium to the plant as the preferred environment. The recently discovered sequence similarities between several putative Hrp proteins and known proteins from other bacteria, however, led to a very different hypothesis, namely, involvement of Hrp proteins in protein secretion. We have

sequenced the entire *hrp* cluster of *X.c. pv. vesicatoria*. Since most *hrp* sequences from this and other bacteria are not yet published, I will summarize our results and refer to the other phytopathogenic bacteria as I go along. Based on genetic analyses and the open reading frames (ORFs) with a high coding probability we predict 21 *hrp* genes in the 25 kb *hrp* cluster of *X.c. pv. vesicatoria*. Their transcriptional organization is depicted in Fig. 1A. The loci *hrpA* and *hrpB* are transcribed from right to left; the other four loci are transcribed from left to right (Schure and Bonas 1992a). According to the locus (*hrpA*-*hrpF*) we have numbered the ORFs consecutively. The *hrpA* locus appears to contain just one *hrp* gene, *hrpA1*. The *hrpB* operon contains eight ORFs, called *hrpB1*-*hrpB8*, etc. A region of about 4 kb between *hrpE* and *hrpF* does not seem to be involved in the interaction with the plant because insertions in this region do not lead to a change in phenotype (Bonas et al. 1991).

What are the characteristics of the Hrp proteins? It should be noted that, except for three proteins, expression of the other 18 has yet to be demonstrated in *X.c. pv. vesicatoria*. A number of putative Hrp proteins are most likely associated with or localized in the bacterial membrane. For example, the HrpC2 protein sequence contains eight transmembrane domains but lacks a signal sequence, suggesting an inner membrane localization (Fenselau et al. 1992). Both HrpA1 and HrpB3 contain an NH<sub>2</sub>-terminal signal sequence and one (HrpA1) or two (HrpB3) transmembrane domains, suggesting that a part of these proteins might be targeted to the outer membrane. The signal sequence of HrpB3 resembles signal peptidase II sequences which are typical of lipoproteins (Fenselau et al. 1992). Experiments using radioactively labeled palmitate are underway to test whether HrpB3 is a lipoprotein. Recently, both HrpB3 and HrpA1 were shown to be localized in the *X.c. pv. vesicatoria* membrane fraction using polyclonal antibodies (S. Fenselau, C. Marie, and U. Bonas, manuscript in preparation). The HrpB6 protein is a putative ATPase with highly conserved nucleotide and magnesium binding domains. It is more similar to protein traffic ATPases than to proton pump ATPases, and the lack of membrane spanning domains suggests a cytoplasmic location (Fenselau et al. 1992).

Searches of the database revealed sequence relatedness of more than half of the *X.c. pv. vesicatoria* Hrp proteins with putative proteins in other bacteria, including different plant pathogens. High DNA sequence identity (more than 90%) was found to a 2.7 kb fragment carrying pathogenicity genes from *X.c. pv. glycines* (Hwang et al. 1992). The authors predicted two ORFs, whereas in *X.c. pv. vesicatoria*, this region contains three ORFs corresponding to the *hrpC3*, *hrpD1* and *hrpD2* genes. Complementation studies indicated that part of the *hrp* region is colinear in the two pathogens of *Xanthomonas* (unpublished).

The deduced amino acid sequences of *hrp* genes published from *P. solanacearum* (Gough et al. 1992, 1993; Genin et al. 1992) show significant similarity to *X.c. pv. vesicatoria* proteins (Table 1; Fig. 1). One exception is the *hrpB* regulatory gene from *P. solanacearum* which is not present in the 25 kb *hrp* region or in the flanking region of the *X.c. pv. vesicatoria hrp* cluster as determined by DNA sequence analysis and hybridization studies (T. Horns and U. Bonas,

unpublished). Furthermore, several of the proteins mentioned are conserved in other species (Fig. 1), however, the degree of sequence similarity varies greatly (Table 1). The HrpA1 protein from *X.c. pv. vesicatoria* is 48% and 29% identical to proteins from *P. solanacearum* (HrpA; Gough et al. 1992) and *P. syringae* (HrpH; Huang et al. 1992), respectively. HrpC2 from *X.c. pv. vesicatoria* is even more conserved, being 66% identical to the corresponding HrpO protein of *P. solanacearum* (Gough et al. 1993), whereas the *hrp* genes from *E. amylovora* (Wei and Beer 1994) and from *P. syringae* (Huang et al. 1993) both show 62% similarity to *hrpC2* from *X.c. pv. vesicatoria*. *P. syringae* also contains a *hrpB3* related gene, called *hrpY*, and a *hrpD2* related gene, *hrpW* (H.-C. Huang, personal communication). Thus, the high degree of DNA sequence conservation that was reported earlier (see above) is also seen on the protein level. It appears that *hrp* genes in *X.c. pv. vesicatoria* are more closely related to *P. solanacearum* than to *P. syringae* and to *Erwinia*. As more and more homologous *hrp* genes are found in other bacteria nomenclature might become confusing. However, as long as the genes have not been shown to be functionally homologous, we will continue to use these names.

Besides genes that are conserved among the major phytopathogenic bacteria some genes are absent in the *hrp* region of more distantly related species. For example, there are no known homologs of the *hrp* genes *hrpN* (Wei et al. 1992a), and *hrpZ* (He et al. 1993) (see below), and of *hrpJ* from *P. syringae* (Huang et al. 1993) in the *X.c. pv. vesicatoria hrp* cluster (unpublished; see Fig. 1). Similarities of 50%-60% were found recently between HrpA1 and HrpB3 from *X.c. pv. vesicatoria* and two putative *Nol* proteins of *Rhizobium fredii* that are encoded by a cultivar specificity region. *NolT* and *NolW* mutants have a wider host range in nodulation of soybean (Meinhardt et al. 1993). In addition, the authors mention that release of proteins is affected.

Last but not least, Table 1 summarizes the significant sequence similarities which have been found to proteins from animal bacterial pathogens. A number of putative Hrp proteins are related to proteins in animal pathogens such as *Salmonella*, *Shigella*, and *Yersinia* ssp. Since the first similarities found were to the Ysc, Vir, and Lcr proteins from *Yersinia* ssp., this group of organisms became a "role model" for plant pathologists (Fenselau et al. 1992; Gough et al. 1992; Huang et al. 1992). In *Yersinia*, these proteins are essential for the secretion of virulence factors, called Yops (*Yersinia* outer protein; Michiels et al. 1990, 1991). Since they are described in detail in the chapter by G.R. Cornelis, I will mention only a few important features. The Yops are hydrophilic proteins that lack a typical NH<sub>2</sub>-terminal signal peptide, and are secreted by using an entirely different pathway from that previously described for protein secretion. The genes involved in secretion are clustered on a 70 kb virulence plasmid. In case of a mutation, e.g., in *YscI*, the Yops accumulate in the cytoplasm (Michiels et al. 1991). Although their direct role in transport has yet to be demonstrated, it is believed that the Ysc and Lcr proteins mentioned in Table 1 are parts of a special transport apparatus for Yop secretion. Similarly, *Shigella flexneri* secretes virulence factors, called Ipa (invasion plasmid antigens), that are distinct from Yops but share the general

Table 1. Sequence similarities of *Xanthomonas campestris* pv. *vesicatoria* Hrp proteins

<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>	HrpA1 <sup>1</sup>	HrpB6 <sup>1</sup>	HrpB3 <sup>1</sup>	HrpC1 <sup>2</sup>	HrpC2 <sup>1</sup>	HrpC3 <sup>2</sup>	HrpD1 <sup>2</sup>	HrpD2 <sup>2</sup>
<i>Pseudomonas solanacearum</i>	HrpA <sup>3</sup> (66%)	HrpE <sup>4</sup>	HrpI <sup>3</sup> (70%)	HrpN <sup>5</sup> (74%)	HrpO <sup>3</sup> (81%)	HpaP <sup>5</sup> (54%)	HrpQ <sup>4</sup>	HrpT <sup>4</sup>
<i>Pseudomonas syringae</i> pv. <i>syringae</i>	HrpH <sup>6</sup> (52%)				HrpI <sup>7</sup> (62%)			
<i>Yersinia enterocolitica</i>	YscC <sup>8</sup> (55%)		YscJ <sup>8</sup> (56%)					
<i>Yersinia pestis</i>	YscC <sup>9</sup> (55%)				LcrD <sup>10</sup> (70%)		LsaA <sup>11</sup> (52%)	LsaB <sup>11</sup> (72%)
<i>Yersinia pseudotuberculosis</i>		YscN <sup>12</sup> (73%)	LcrKa <sup>13</sup> (56%)					
<i>Shigella flexneri</i>	MxiD <sup>14</sup> (50%)	Spa47 <sup>15</sup> (65%)	MxiJ <sup>16</sup> (52%)	Spa40 <sup>17</sup> (55%)	MxiA <sup>18</sup> (65%)			Spa24 <sup>15</sup> (67%)
<i>Salmonella typhimurium</i>	InvG <sup>19</sup> (52%)	SpaL <sup>20</sup> (70%)		SpaS <sup>20</sup> (56%)	InvA <sup>22</sup> (67%)			SpaP <sup>20</sup> (64%)
		FliH <sup>21</sup> (65%)						
<i>Bacillus subtilis</i>		FlaA-ORF4 <sup>22</sup> (68%)		FliH <sup>24</sup> (62%)	FliA <sup>25</sup> (63%)			FliP <sup>26</sup> (68%)
<i>Escherichia coli</i>		β-F1 <sup>27</sup> (53%)						FliP <sup>28</sup> (65%)
<i>Erwinia carotovora</i>							MopB <sup>29</sup> (49%)	MopC <sup>29</sup> (65%)
<i>Erwinia amylovora</i>					HrpI <sup>30</sup> (62%)			
<i>Rhizobium fredii</i>	NolW <sup>31</sup> (51%)		NolT <sup>31</sup> (61%)					
<i>Caulobacter crescentus</i>					FibF <sup>32</sup> (55%)			

Similarities between deduced amino acid sequences of Hrp proteins from *X.c.pv.vesicatoria* and other proteins include conservative amino acid exchanges. Number in parentheses indicates percent similarity.

Superscript numbers indicate references as follows:

1, FENSELAW et al. 1992; 2, Bonas et al., unpublished; 3, GOUGH et al. 1992; 4, GENIN et al. 1993, sequences unpublished; 5, GOUGH et al. 1993; 6, HUANG et al. 1992; 7, HUANG et al. 1993; 8, MICHELS et al. 1991; 9, HADDIX and STRALEY 1992; 10, PLANO et al. 1991; 11, Fields et al. unpublished, accession # L22495; 12, Galyov, unpublished, accession # U00998; 13, RIMPIAINEN et al. 1992; 14, ALLAOUI et al. 1993; 15, VENKATESAN et al. 1992; 16, ALLAOUI et al. 1992; 17, SASAKAWA et al. 1993; 18, ANDREWS and MAURELLI 1992; 19, Lodge et al., unpublished, accession # X75302; 20, GROISMAN and OCHMAN 1993; 21, VOGLER et al. 1991; 22, GALAN et al. 1992; 23, ALBERTINI et al. 1991; 24, Carpenter et al., unpublished, accession # X741212; 25, CARPENTER and ORDAL 1993; 26, BISCHOFF et al. 1992; 27, SARASTE et al. 1981; 28, MALAKOOTI et al. unpublished, accession # L21994; 29, MULHOLLAND et al. 1993; 30, WEI and BEER 1993; 31, MEINHARDT et al. 1993; 32, RAMAKRISHNAN et al. 1991; SANDERS et al. 1992.





### 5.3 *PopA* from *Pseudomonas solanacearum*

An HR-inducing protein has been identified and characterized from *P. solanacearum* culture supernatants, called Pop (Pseudomonas out protein; Arlat et al. 1994). PopA1 and two shorter derivatives, PopA2 and PopA3, induce the HR in tobacco and in certain, but not all, *Petunia* lines. Like the harpins, the Pop proteins are also heat-stable and glycine-rich, however, the sequence is entirely different. In contrast to the harpins, the *popA* gene is not a *hrp* gene but is located outside of the large *hrp* cluster. Interestingly, expression of *popA* is *hrpB*-dependent, i.e., the gene is part of the *hrp* regulon. Mutations in *popA* do not affect the HR on tobacco or pathogenicity on tomato suggesting that more than one HR-inducing factor is produced. Arlat et al. (1994) convincingly showed that secretion of PopA is dependent on other *hrp* genes, such as *hrpA*, *hrpN*, and *hrpO* (Fig. 1B). If a bacterial strain virulent towards *Petunia* is found it will be interesting to see if PopA acts as an avirulence protein in *Petunia* as has been suggested by the authors.

These exciting findings prove that certain Hrp proteins of *P. s. pv. syringae* and *P. solanacearum* play a role in transport of HR elicitors (Fig. 2). They also stimulate more questions. It needs to be shown that harpins and PopA are in fact secreted when the bacteria interact with the plant (the *hrp* genes were induced in vitro). Are harpins conserved among pathogens of *P. syringae*? How many elicitors of the non-host HR in tobacco can be found? Is the mechanism of recognition in tobacco identical with the *Erwinia* and *P. s. pv. syringae* harpins and the *P. solanacearum* Pops?

### 6 Regulation of Expression of *hrp* Genes

Expression of *hrp* genes is controlled by environmental conditions and has been studied on the RNA level as well as using transcriptional fusions to reporter genes such as the *E. coli* genes encoding  $\beta$ -galactosidase or  $\beta$ -glucuronidase. In general, expression of *hrp* loci is not detectable when the bacteria are grown in complex culture media. However, after growth of the bacteria in the plant, *hrp* genes are expressed. Attempts to mimic the conditions that the different bacterial species encounter in the plant tissue resulted in the finding that growth in minimal media without any plant-derived factor was sufficient to induce *hrp* genes. This has led to the speculation that the bacteria have to experience some kind of starvation conditions for full expression of *hrp* genes. One of the first indications for *hrp* gene expression in vitro, and clearly a breakthrough, was a report on the *hrp*-dependent expression of an avirulence gene from the soybean pathogen *P. s. pv. glycines* (Huvni et al. 1989).

Since the composition of minimal media differs depending on the bacterium studied, the most important findings will be summarized for representative pathogens. Parameters like carbon source, concentration of organic nitrogen and phosphate, osmolarity, and pH have been found to be important. High con-

centration of organic nitrogen generally appears to suppress *hrp* gene activation. Only two regulatory genes have been studied so far (see below). Interestingly, they both belong to different families of regulatory proteins.

#### 6.1 *Pseudomonas syringae*

Expression of all seven *hrp* loci in the large cluster of *P. s. pv. phaseolicola* is suppressed in complex medium but induced in the plant. Induction occurs in the susceptible host plant as well as in the non-host, tobacco, suggesting that there is no plant species-specific molecule involved in control of host range (Rahme et al. 1992). Five complementation groups, *hrpAB*, *hrpC*, *hrpD*, *hrpE* and *hrpF*, can also be induced in M9 minimal medium containing sucrose as a carbon source, however, induction is affected by pH, osmolarity, and carbon source, and never reaches the levels obtained in the plant (Rahme et al. 1992). A similar observation was made earlier for the avirulence gene *avrB* in *P. s. pv. glycines*. Induction occurred in a minimal medium containing fructose, mannitol, or sucrose. Expression of *avrB* is dependent on *hrp* genes homologous to *hrpRS* and *hrpL* from *P. s. pv. phaseolicola* and was suppressed by TCA cycle intermediates such as citrate and succinate (Huvni et al. 1989). *hrp* gene expression in *P. s. pv. syringae* occurs in the same medium as described by Huvni et al. (1989); (Thang et al. 1991; Xiao et al. 1992). The authors showed *hrp* gene induction in the non-host plant, tobacco, but no data for the host plant. The *P. s. pv. phaseolicola* loci *hrpL* and *hrpRS* are only expressed to a very low level in M9 minimal medium and are induced at least 1000-fold when the bacteria are inoculated into the plant. This led to the conclusion that, at least for expression of *hrpL* and *hrpRS*, specific plant factors might be necessary (Rahme et al. 1992).

#### 6.2 Regulatory Genes *hrpRS* and *rpoh* of *Pseudomonas syringae* pv. *phaseolicola*

The results on environmental factors inducing or suppressing *hrp* gene expression suggested that specific regulatory genes are involved in the control of *hrp* promoter activities. At least two loci are involved in positive regulation of the other *hrp* loci of *P. s. pv. phaseolicola* *hrp* cluster (Fellay et al. 1991). While there is no information published for *hrpL*, *hrpRS* has been sequenced. It contains two genes whose predicted protein products are 60% identical to each other (Grimm and Pamosouros 1989; Miller et al. 1993). The HrpS protein is similar to members of the NtrC family of regulatory proteins in gram-negative bacteria. Most NtrC-like regulatory proteins are members of two-component systems, with a sensor protein that in turn activates a response element by phosphorylation of a site in the conserved NH<sub>2</sub>-terminal domain (Alamgir et al. 1989). The putative sensor component operating in *hrp* gene regulation has not been identified. It is postulated that HrpS is the activating protein, however, direct biochemical data

have not been presented. The lack of a typical  $\text{NH}_2$ -terminal domain in HrpS could indicate that a different mechanism may be involved in HrpS activation. Apparently, *hrpS*-related sequences are also present in other bacteria, e.g., in *P. s. syringae* (Heu and Hurcheson 1993) and in *Erwinia amylovora* (Beer et al. 1993). *E. stewartii* contains a transcriptional regulator, *WtsA*, with 52% identity to HrpS of *P. s. phaseolicola*. The *hrpS* clone, however, was unable to functionally complement a *wtsA* mutant (Friedenick et al. 1993).

The structure of the *hrpRS* locus and the finding of -24/-12 consensus sequences upstream of *hrpRS* indicated a possible role in transcriptional activation for transcription factor sigma 54, encoded by *spoN* (Gamm and Parnopoulos 1989). In a preliminary report, Feuley et al. (1991) demonstrated that *hrp* gene expression in *P. s. pv. phaseolicola* is indeed dependent on *spoN*. A *spoN* mutant of *P. s. pv. phaseolicola* is a glutamine auxotroph and nonpathogenic. Whether *spoN* is generally involved in regulation of *hrp* gene expression is not clear. In *X. c. pv. vesicatoria*, *spoN* is clearly not involved in *hrp* gene expression and pathogenicity (T. Horns and U. Bonas, manuscript in preparation).

Recently, Müller et al. (1993) have reported the identification of two new loci, *hrpQ* and *hrpT*, from *P. s. pv. phaseolicola* that affect activation of *hrpRS* in *trans*. However, since *hrpRS* is strongly induced in plants while both *hrpQ* and *hrpT* are constitutively expressed, there must be more factors involved in *hrp* gene regulation. Strains carrying mutations in either *hrpQ* or *hrpT* are amino acid auxotrophs (methionine and tryptophan). *hrpQ* and *hrpT* are probably involved in methionine and tryptophan biosynthesis, respectively (Müller et al. 1993). As stated above, such mutants would normally have been eliminated from the *hrp* mutant analysis.

### 6.3 Conserved Sequence Boxes in *Pseudomonas syringae*

A conserved sequence, the so-called harp box (TGAGCAAGC, Fittau et al. 1991), upstream of four *hrp* loci in *P. s. pv. phaseolicola*, was suggested to be involved in positive regulation of expression. Similar motifs were described for the promoter regions of several *P. syringae* avirulence genes, the expression of which is dependent on *hrpRS* and on *spoN* (Huvn et al. 1989; Salmeron and Straskawicz 1993; Innes et al. 1993; Shen and Keen 1993). These studies led to a revised 'harp' box sequence (GGAAACCA). Its significance in protein binding has not been shown but *avrD* promoter constructs lacking the harp box are no longer inducible (Shen and Keen 1993). A harp box-related motif was also found upstream of transcription unit 3 in *P. solanacearum* (Gough et al. 1993).

There is no harp box sequence in *Xanthomonas* *hrp* gene promoters. Another sequence motif that occurs in the promoter region of *hrp* loci in *X. c. pv. vesicatoria* was recently identified. This "PIP" (plant-inducible promoter) box has the sequence TTGGC-N15-TTGGC and occurs upstream of the -35 consensus sequence in four out of six *hrp* promoters (S. Fenselau and U. Bonas, unpublished). Experiments are underway to test whether this is a protein binding motif.

### 6.4 *Xanthomonas campestris*

Expression of *hrp* genes in *X. c. pv. campestris* was determined after growth in vitro and found to be induced in a minimal medium with sucrose and/or fructose as carbon source. No expression occurred in complex media or with high concentrations of organic nitrogen (Arlat et al. 1991). In *X. c. pv. vesicatoria*, expression of the six *hrp* loci is induced in the plant but cannot be efficiently induced in the synthetic media tested so far. However, culture filtrates of sterile tomato cell suspension cultures (called TCM) induced expression of the six *hrp* loci in *X. c. pv. vesicatoria* whereas the basal Murashige-Skoog culture medium did not. The inducing factor(s) could only partially be purified from TCM and was found to be smaller than 1000 dalton, heat-stable, organic, and hydrophilic (Schulte and Bonas 1992a). De novo transcription of all *hrp* loci occurs rapidly within 1 h after transfer of the bacteria into TCM (S. Fenselau and U. Bonas, unpublished). A minimal medium was designed which would not suppress *hrp* gene induction. This medium, called XVM1, induces the *hrpF* locus (Fig. 1A) to high levels and differs from the other media described above, in particular by its low concentration in phosphate. Both sucrose and methionine are needed for efficient induction. It is not known whether a plant factor is necessary for activation of the other *hrp* loci, or if the XVM1 medium still lacks components or contains them in suppressing amounts (Schulte and Bonas 1992b).

### 6.5 *Erwinia* and *Pseudomonas solanacearum*

The *hrp* genes of *Erwinia amylovora* are rapidly induced in the non-host, tobacco, and more slowly in the host, pear. Several loci were induced in minimal medium with mannitol as a carbon source. Induction was suppressed by high concentrations of nitrogen and by glucose and was slightly temperature dependent (Weir et al. 1992b).

In *P. solanacearum*, the *hrp* cluster was also induced in host and in non-host plants, as well as in minimal medium. The best carbon sources for induction of four of the six transcription units were pyruvate and glutamate while, as in other bacteria, casamino acids suppressed induction (Arlat et al. 1992). The two rightmost *hrp* transcription units (5 and 6; Fig. 1B) are constitutively expressed but can be induced under certain conditions (Gemin et al. 1992).

The only other gene reported to regulate *hrp* gene expression is *hrpB* from *P. solanacearum*. The gene is part of the *hrp* cluster and appears to be a member of the AraC family of positive regulatory proteins. Interestingly, *hrpB* is related to *vif* of *Yersinia* (Coinneus et al. 1989; Gemin et al. 1992). The *hrpB* gene positively regulates four of the six *hrp* loci, as well as the *popA* locus, located outside of the *hrp* cluster which encodes a protein secreted in a Hrp-dependent way (see above; Arlat et al. 1994). Whether the HrpB protein binds directly to *hrp* promoters is not yet known.

At this time one can only speculate whether the regulatory systems for *hrp* gene expression employed by *P. solanacearum* and *P. syringae* are really different

or whether there is a global regulatory network thus allowing the fine tuning of gene expression in response to environmental cues. In conclusion, most *htrp* loci from different bacteria are inducible in a particular minimal medium. At this time it cannot be ruled out that stimulation of *htrp* gene expression involves specific plant factors as was described for the virulence genes of *Agrobacterium* (Wians 1992). Since the composition of the nutrients available to the pathogen in the plant is not known one can only speculate that the conditions described above reflect the situation in the plant. It is noteworthy that the *in vitro* culture will only mimic the dynamic nutritional situation that bacteria experience in their interaction with a plant for a short time. In mammalian bacterial pathogens, the expression of genes involved in virulence is also regulated in response to environmental cues rather than to specific host molecules. This subject has been reviewed recently (Mekalanos 1992 and in accompanying chapters), and I will only mention some important factors. In *Yersinia*, the *vir* and *lcr* genes are regulated by low calcium (low calcium response genes; Straley et al. 1993) and by temperature (Connors et al. 1989; see chapter by Connors). A calcium effect has not been described for any plant bacterium. In our laboratory no effect of calcium on *htrp* gene expression in XVM1 was observed (Schulte and U. Bonas, unpublished). Expression of *invA* of *S. typhimurium* of the *mxl* and *ipa* genes of *Shigella* is affected by osmolality and the later genes also by temperature (Galan and Curtiss 1990; Hale 1991).

**Acknowledgements.** I thank my previous and present coworkers—Ilse Balbo, Martina Gutscow, Stefan Fenslau, Torsten Thoms, Corinne Marie, Michèle Pierre, Ralf Schulte, and Kai Wengenik—for contributing to the data described here and for fruitful discussions. I am also grateful to my colleagues for sending preprints and sharing unpublished results, and to Heather McKinnon and John Mansfield for helpful suggestions on the manuscript. The research in my laboratory was supported in part by grants from the Bundesministerium für Forschung und Technologie (322-4-003/0316300A), the Deutsche Forschungsgemeinschaft, and the FEC (BIO1-C190-0168).

## References

- Albertini AM, Caranton I, Cabb WD, Scollone F, Ghitzi A (1991) The *ltaA* locus of *Bacillus subtilis* is part of a large operon coding for flagellar structures, motility functions, and an ATPase-like polypeptide. *J Bacteriol* 173: 3573-3579
- Albright LM, Huale E, Ausubel FM (1989) Prokaryotic signal transduction mediated by sensor and regulator protein pairs. *Annu Rev Genet* 23: 311-336
- Allout A, Sansonetti PJ, Pissot C (1992) *MxiJ*, a lipoprotein involved in secretion of *Shigella* *ipa* invasins, is homologous to *YnfJ*, a secretion factor of the *Yersinia* Yop proteins. *J Bacteriol* 174: 7651-7653
- Altman A, Sansonetti PJ, Pissot C (1992) *MxiD*, an outer membrane protein necessary for the secretion of the *Shigella* *flexneri* *ipa* invasins. *Mol Microbiol* 7: 59-68
- Andersson GP, Maurer AT (1992) *mxlA* of *Shigella flexneri* 2a, which facilitates export of invasion plasmid antigens, encodes a homolog of the low-calcium response protein, *LcrD*, of *Yersinia* *pestis*. *Infect Immun* 60: 3287-3295
- Almt M, Gough CL, Barber CE, Boucher CA, Daniels MJ (1991) *Xanthomonas campestris* contains a cluster of *htrp* genes related to the *htrp* cluster of *Pseudomonas solanacearum*. *Mol Plant Microbe Interact* 4: 593-601
- Almt M, Gough CL, Zischek C, Barberis PA, Triguel A, Boucher CA (1992) Transcriptional organization and expression of the large *htrp* gene cluster of *Pseudomonas solanacearum*. *Mol Plant Microbe Interact* 5: 187-193
- Almt M, Van Gijsegem F, Huet JC, Pernellet JC, Boucher CA (1994) *Poga1*, a protein which induces a hypersensitive like response on specific *Petunia* genotypes, is secreted via the *Hrp* pathway of *Pseudomonas solanacearum*. *EMBO J* 13: 543-553
- Barry MA, Guinebrelle MH, Marcias B, Coissac A, Paulin JP, Laurent J (1990) Cloning of a large gene cluster involved in *Erwinia amylovora* CFBP1430 virulence. *Mol Microbiol* 4: 777-787
- Bauer DW, Beer SV (1991) Further characterization of an *htrp* gene cluster of *Erwinia amylovora*. *Mol Plant Microbe Interact* 4: 483-489
- Beer SV, Bauer DW, Jiang X, Leby RJ, Sneath BJ, Wei ZM, Wilcox DA, Zumoff CH (1991) The *htrp* gene cluster of *Erwinia amylovora*. In: Henneke H, Verma DPS (eds) *Advances in molecular genetics of plant-microbe interactions*, vol 1. Kluwer Academic, Dordrecht, Netherlands, pp 53-60
- Beer SV, Wei ZM, Leby RJ, He SY, Bauer DW, Colmer A, Zumoff C (1993) *htrp* has unique universal elicitors of the hypersensitive response of phytopathogenic bacteria? In: Neister EW, Verma DPS (eds) *Advances in molecular genetics of plant-microbe interactions* vol 2. Kluwer Academic, Dordrecht, Netherlands, pp 281-286
- Bischoff DS, Weinreich MR, Odel GW (1992) Nucleotide sequence of *Bacillus subtilis* flagellar biosynthetic genes *flp* and *flhQ* and identification of a novel flagellar gene. *J Bacteriol* 174: 4017-4025
- Bonas U, Schulte R, Fenslau S, Minsavage GV, Staskawicz BJ, Stall RE (1991) Isolation of a gene cluster from *Xanthomonas campestris* pv. *vesicatoria* that determines pathogenicity and the hypersensitive response on pepper and tomato. *Mol Plant Microbe Interact* 4: 81-88
- Boucher CA, Van Gijsegem F, Barberis PA, Almt M, Zischek C (1992) *Pseudomonas solanacearum* genes controlling both pathogenicity and hypersensitivity on tobacco are clustered. *J Bacteriol* 169: 5626-5632
- Boucher CA, Gough CL, Almt M (1992) Molecular genetics of pathogenicity determinants of *Pseudomonas solanacearum* with special emphasis on *htrp* genes. *Annu Rev Phytopathol* 30: 443-461
- Curpenter PG, Odel GW (1993) *Bacillus subtilis* *FlhA*, a flagellar protein related to a new family of signal-transducing receptors. *Mol Microbiol* 7: 735-743
- Coplin DL, Frederick RD, Majerczak DR, Tuttle LD (1992) Characterization of a gene cluster that specifies pathogenicity in *Erwinia stewartii*. *Mol Plant Microbe Interact* 5: 81-88
- Cornelis G, Stuijers C, Lambert de Rouvroit C, Michiels T (1993) Homology between *VirF*, the transcriptional activator of the *Yersinia* virulence regulon, and *AraC*, the *Escherichia coli* arabinose operon regulator. *J Bacteriol* 171: 254-262
- Fellay R, Rahme LG, Mindrinos MN, Frederick RD, Pisi A, Panopoulos NJ (1991) Genes and signals controlling the *Pseudomonas syringae* pv. *phaseolicola*-plant interaction. In: Henneke H, Verma DPS (eds) *Advances in molecular genetics of plant-microbe interactions* vol 1. Kluwer Academic, Dordrecht, Netherlands, pp 45-52
- Fenslau S, Balbo I, Bonas U (1992) Determinants of pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. *Mol Plant Microbe Interact* 4: 593-601
- Frederick RD, Majerczak DR, Coplin DL (1993) *Erwinia stewartii* *WiscA*, a positive regulator of pathogenicity gene expression, is similar to *Pseudomonas syringae* pv. *phaseolicola* *HrpS*. *Mol Microbiol* 9: 477-485
- Galan JE, Curtiss III (1990) Expression of *Salmonella typhimurium* genes required for invasion is regulated by changes in DNA supercoiling. *Infect Immun* 58: 1879-1885
- Galan JE, Gnocchio C, Costales P (1992) Molecular and functional characterization of the *Salmonella* invasion gene *invA*: homology of *invA* to members of a new protein family. *J Bacteriol* 174: 4338-4349
- Gann S, Gough CL, Zischek C, Boucher CA (1992) The *htrp* gene encodes a putative regulator of pathogenicity genes from *Pseudomonas solanacearum*. *Mol Microbiol* 6: 3005-3016
- Gann S, Gough CL, Almt M, Zischek C, Van Gijsegem F, Barberis PA, Boucher CA (1993) Involvement of *Pseudomonas solanacearum* *htrp* genes in the secretion of a bacterial compound which induces a hypersensitive-like response on tobacco. In: Neister EW, Verma DPS (eds) *Advances in molecular genetics of plant-microbe interactions*, vol 2. Kluwer Academic, Dordrecht, Netherlands, pp 259-266
- Gogen JD, Voiter J, Straley SC (1984) Genetic analysis of the low calcium response in *Yersinia* *pestis* *Mu* d1 *Ap* *htrp* insertion mutants. *J Bacteriol* 160: 842-848
- Gough CL, Gann S, Zischek C, Boucher CA (1992) *htrp* genes of *Pseudomonas solanacearum* are homologous to pathogenicity determinants of animal pathogenic bacteria and are conserved among plant pathogenic bacteria. *Mol Plant Microbe Interact* 5: 384-389
- Gough CL, Gann S, Lopes V, Boucher CA (1993) Homology between the *HrpD* protein of *Pseudomonas*

- solanaceum and bacterial proteins implicated in a signal peptide independent secretion mechanism. *Mol Gen Genet* 239: 378-392
- Grimm C, Panopoulos NJ (1989) The predicted product of a pathogenicity locus from *Pseudomonas syringae* pv. phaseolicola is homologous to a highly conserved domain of several prokaryotic regulatory proteins. *J Bacteriol* 171: 5031-5038
- Groisman EA, Ochman H (1983) Cognate gene clusters govern invasion of host epithelial cells by *Salmonella typhimurium* and *Shigella flexneri*. *EMBO J* 12: 3779-3787
- Haddix PL, Straley SC (1992) Structure and regulation of the *Yersinia pestis* yscBCDEF operon. *J Bacteriol* 174: 4870-4878
- Heib J (1991) Genetic basis for virulence in *Shigella* species. *Microbiol Rev* 55: 206-224
- He SY, Huang HC, Collier A (1993) *Pseudomonas syringae* pv. *syringae* hcrp<sub>hnp</sub>: a protein that is secreted via the hnp pathway and elicits the hypersensitive response in plants. *Cell* 73: 1255-1266
- Hau S, Hutcheson SW (1993) Nucleotide sequence and properties of the hrmA locus associated with the *Pseudomonas syringae* pv. *syringae* 61 hnp gene cluster. *Mol Plant Microbe Interact* 6: 553-564
- Huang HC, Schuurink R, Denny TP, Atkinson MM, Baker CJ, Yocel I, Hutcheson SW, Collier A (1988) Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco plants. *J Bacteriol* 170: 4748-4756
- Huang Y, Xu P, Serrano L (1990) A second cluster of genes that specify pathogenicity and host response in *Pseudomonas solanacearum*. *Mol Plant Microbe Interact* 3: 48-53
- Huang YC, Hutcheson SW, Collier A (1991) Characterization of the hnp cluster from *Pseudomonas syringae* pv. *syringae* 61 and *Trifolium* tagging of genes encoding exported or membrane-spanning hnp proteins. *Mol Plant Microbe Interact* 4: 469-476
- Huang YC, He SY, Bauer DW, Collier A (1992) The *Pseudomonas syringae* pv. *syringae* 61 hnp product, an envelope protein required for elicitation of the hypersensitive response in plants. *J Bacteriol* 174: 6878-6885
- Huang YC, Xiao Y, Lin RH, Lu Y, Hutcheson SW, Collier A (1993) Characterization of the *Pseudomonas syringae* pv. *syringae* hnpJ and hnpI genes: homology of hnpI to a superfamily of proteins associated with protein translocation. *Mol Plant Microbe Interact* 6: 515-520
- Huynh TV, Dahlbeck D, Staskiewicz BJ (1989) Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. *Science* 245: 1374-1377
- Hwang I, Lim SM, Shaw PD (1992) Cloning and characterization of pathogenicity genes from *Xanthomonas campestris* pv. *glyceris*. *J Bacteriol* 174: 1923-1931
- Innes RW, Bent AF, Kunkel BN, Bisgrove SN, Staskiewicz BJ (1993) Molecular analysis of avirulence gene avrP2 and identification of a putative regulatory sequence common to all known *Pseudomonas syringae* avirulence genes. *J Bacteriol* 175: 4859-4869
- Kamdar HV, Kamoun S, Kado CI (1993) Restoration of pathogenicity of avirulent *Xanthomonas oryzae* pv. *oryzae* and *X. campestris* pathways by reciprocal complementation with the hrxX and hnpX genes and identification of hnpX function by sequence analyses. *J Bacteriol* 175: 2017-2025
- Kamoun S, Kado CI (1990) A plant-inducible gene of *Xanthomonas campestris* pv. *campestris* encodes an extracellular component required for growth in the host and hypersensitivity on nonhosts. *J Bacteriol* 172: 5165-5172
- Kamoun S, Kamdar HV, Tolia E, Kado CI (1992) Incompatible interactions between cucurbers and *Xanthomonas campestris* involve a vascular hypersensitive response: role of the hnpX locus. *Mol Plant Microbe Interact* 5: 22-33
- Klement Z (1982) Hypersensitivity in: Mount MS, Lacey GH (eds) *Phytopathogenic prokaryotes*, vol 2. Academic Press, New York, pp 149-177
- Laby RJ, Beer SV (1992) Hybridization and functional complementation of the hnp gene cluster from *Erwinia amylovora* strain Ea321 with DNA of other bacteria. *Mol Plant Microbe Interact* 5: 412-419
- Lambert de Rouvroil C, Stiers C, Cornelis GR (1992) Role of the transcriptional activator, Ynf, and temperature in the expression of the pYV plasmid genes of *Yersinia enterocolitica*. *Mol Microbiol* 6: 395-409
- Leite RP, Minsavage GV, Bonas U, Stall RF (1994) Detection and identification of plant pathogenic strains of *Xanthomonas* based on amplification of DNA sequences related to the hnp genes of *Xanthomonas campestris* pv. *vesicatoria*. *Appl Environ Microbiol* 60: 1058-1077
- Lindgren PB, Patel RC, Panopoulos NJ (1986) Gene cluster of *Pseudomonas syringae* pv. *phaseolicola* controls pathogenicity on bean plants and hypersensitivity on nonhost plants. *J Bacteriol* 168: 517-522
- Lindgren PB, Panopoulos NJ, Staskiewicz BJ, Dahlbeck D (1988) Genes required for pathogenicity and hypersensitivity are conserved and interchangeable among pathways of *Pseudomonas syringae*. *Mol Gen Genet* 211: 499-506
- Lindsay WP, Lamb CJ, Dixon RA (1993) Microbial recognition and activation of plant defense systems. *Trends Microbiol* 1: 181-187
- Loudens I, Debussche L, Bion A, Lacroix J-M, Bohin JP (1993) Homology between a genetic locus (hnpA) involved in the osmoregulated biosynthesis of periplasmic glucans in *Escherichia coli* and a genetic locus (hnpM) controlling pathogenicity of *Pseudomonas syringae*. *Mol Microbiol* 10: 329-340
- Mekalanos JJ (1992) Environmental signals controlling expression of virulence determinants in bacteria. *J Bacteriol* 174: 1-7
- Meinhart LW, Krishnan HB, Balatti PA, Puopple SG (1993) Molecular cloning and characterization of a sym plasmid locus that regulates cultivar-specific nodulation of soybean by *Rhizobium fredii* USDA257. *Mol Microbiol* 9: 17-29
- Michiels T, Wautou P, Basseur R, Ruyschaert JM, Cornelis G (1990) Secretion of Yop proteins by *Yersinia*. *Infect Immun* 58: 2840-2849
- Michiels T, Vanooteghem J-C, Lambert de Rouvroil C, China B, Gustin A, Boudry P, Cornelis GR (1991) Analysis of virC, an operon involved in the secretion of Yop proteins by *Yersinia enterocolitica*. *J Bacteriol* 173: 4994-5009
- Miller W, Mindros MS, Rahme LG, Frederick RD, Grimm C, Gressman R, Kyriakides X, Kottinidis M, Panopoulos NJ (1993) *Pseudomonas syringae* pv. *phaseolicola*-plant interactions: host-pathogen signaling through cascade control of hnp gene expression. In: Nester EW, Verma DPS (eds) *Advances in molecular genetics of plant-microbe interactions*, vol 2. Kluwer Academic, Dordrecht, pp 267-274
- Mukhopadhyay P, Williams J, Mills D (1988) Molecular analysis of a pathogenicity locus in *Pseudomonas syringae* pv. *syringae*. *J Bacteriol* 170: 5479-5488
- Mutlind V, Hinton JCD, Sidebottom J, Toth IK, Hyman LJ, Peromblanc MCW, Reeves PJ, Salmond GPC (1993) A pleiotropic reduced virulence (Rv<sup>-</sup>) mutant of *Erwinia carotovora* subspecies *atroseptica* is defective in flagella assembly proteins that are conserved in plant and animal bacterial pathogens. *Mol Microbiol* 9: 343-356
- Nepold F, Anderson D, Mills D (1985) Cloning determinants of pathogenesis from *Pseudomonas syringae* pv. *syringae*. *Proc Natl Acad Sci USA* 82: 406-410
- Piano GV, Baye SS, Straley SC (1991) LcrD, a membrane-bound regulator of the *Yersinia pestis* low-calcium response. *J Bacteriol* 173: 7293-7303
- Rahme LG, Mindros MN, Panopoulos NJ (1991) Genetic and transcriptional organization of the hnp cluster of *Pseudomonas syringae* pv. *phaseolicola*. *J Bacteriol* 173: 575-586
- Rahme LG, Mindros MN, Panopoulos NJ (1992) Plant and environmental sensory signals control the expression of hnp genes in *Pseudomonas syringae* pv. *phaseolicola*. *J Bacteriol* 174: 3499-3507
- Ramakrishnan G, Zhao J-L, Newton A (1991) The cell cycle-regulated flagellar gene flhF of *Caulobacter crescentius* is homologous to a virulence locus (lcrD) of *Yersinia pestis*. *J Bacteriol* 173: 7283-7292
- Rimpiläinen M, Forsberg A, Wolf-Watz H (1992) A novel protein, LcrQ, involved in the low-calcium response of *Yersinia pseudotuberculosis* shows extensive homology to YopI. *J Bacteriol* 174: 3355-3363
- Salmeron JM, Staskiewicz BJ (1993) Molecular characterization and hnp dependence of the avirulence gene avrPto from *Pseudomonas syringae* pv. *tomato*. *Mol Gen Genet* 239: 6-16
- Sanders LA, Van Way S, Mullin DA (1992) characterization of the *Caulobacter crescentius* flhF promoter and identification of the inferred flhF product as a homolog of the LcrD protein from a *Yersinia enterocolitica* virulence plasmid. *J Bacteriol* 174: 857-866
- Saizae M, Gay NJ, Eberle A, Runswick MJ, Walker JE (1981) The alp operon: nucleotide sequences of the genes for the a, b, and g subunits of *Escherichia coli* ATP synthase. *Nucleic Acids Res* 9: 5287-5296
- Sasakawa C, Komatsu K, Tobe T, Suzuki T, Yoshikawa M (1993) Eight genes in region 5 that form an operon are essential for invasion of epithelial cells by *Shigella flexneri*. *J Bacteriol* 175: 2334-2346
- Schulte R, Bonas U (1992a) Expression of the *Xanthomonas campestris* pv. *vesicatoria* hnp gene cluster, which determines pathogenicity and hypersensitivity on pepper and tomato, is plant inducible. *J Bacteriol* 174: 815-823
- Schulte R, Bonas U (1992b) A *Xanthomonas* pathogenicity locus is induced by sucrose and sulfur-containing amino acids. *Plant Cell* 4: 79-86
- Shen H, Krenn NI (1993) Characterization of the promoter of avirulence gene D from *Pseudomonas syringae* pv. *tomato*. *J Bacteriol* 175: 5916-5924

- Stall RE, Minsavage GV (1990) The use of *htrp* genes to identify opportunistic xanthomonads. In: Klement Z (ed) plant pathogenic bacteria, proceedings of the 7th international conference of plant pathogenic bacteria, Budapest, Hungary, 1989. Akadémiai Kiadó, Budapest, pp 369-374
- Steinberger EM, Beer SV (1988) Creation and complementation of pathogenicity mutants of *Erwinia amylovora*. Mol Plant Microbe Interact 1: 135-144
- Straley SC, Plano GV, Strzypek E, Haddix PL, Fields KA (1993) Regulation by  $Ca^{2+}$  in the *Yersinia low-Ca<sup>2+</sup>* response. Mol Microbiol 8: 1005-1010
- Van Gijsegem F, Genin S, Boucher C (1993) Conservation of secretion pathways for pathogenicity determinants of plant and animal bacteria. Trends Microbiol 1: 175-180
- Verkatesan MM, Buyse JM, Oaks EV (1992) Surface presentation of *Shigella flexneri* invasion plasmid antigens requires the products of the *spa* locus. J Bacteriol 174: 1990-2001
- Vogler AP, Homma M, Inokura VM, Macneil RM (1991) *Salmonella typhimurium* mutants defective in flagellar filament regrowth and sequence similarity of Fli to FliF, vacuolar, and archaebacterial ATPase subunits. J Bacteriol 173: 3564-3572
- Walters K, Maroofi A, Hitchin E, Mansfield J (1990) Gene for pathogenicity and ability to cause hypersensitive reaction cloned from *Erwinia amylovora*. Physiol Mol Plant Pathol 36: 509-521
- Waney VR, Kingsley MT, Gabriel DW (1991) *Xanthomonas campestris* pv. *translucens* genes determining host-specific virulence and general virulence on cereals identified by Tn5-gusA insertion mutagenesis. Mol Plant Microbe Interactions 4: 623-627
- Wei ZM, Leiby RJ, Zurnoff CH, Bauer DW, He SY, Collier A, Beer SV (1992a) Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science 257: 85-88
- Wei ZM, Sneath BJ, Beer SV (1992b) Expression of *Erwinia amylovora htrp* genes in response to environmental stimuli. J Bacteriol 174: 1875-1882
- Wei ZM and Beer SV (1993) Hrp of *Erwinia amylovora* functions in secretion of harpin and is a member of a new protein family. J Bacteriol 175: 7958-7967
- Willis DK, Rich JJ, Habak EM (1991) *htrp* genes of phytopathogenic bacteria. Mol Plant Microbe Interact 4: 132-138
- Wilkins SC (1992) Two way chemical signaling in *Agrobacterium*-plant interactions. Microbiol Rev 56: 12-31
- Xiao YX, Lu Y, Heu SG, Hutchesson SW (1992) Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 *htrp* cluster. J Bacteriol 174: 1734-1741

## The Enigmatic Avirulence Genes of Phytopathogenic Bacteria

J.L. DANGL

1	Action at the "Pathogenic Cusp" .....	99
2	Avirulence Genes as Determinants of Host Range Both Within and Across Species .....	100
3	Virulence Functions of Avirulence Genes .....	105
4	Regulation and Organization of Avirulence Genes .....	108
5	Modes of Avirulence Gene Action .....	110
5.1	The <i>avrD</i> Elicitor .....	110
5.2	The Structural Conundrum of the <i>avrBs3</i> Gene Family .....	111
6	Other Genes Influencing Host Range .....	112
7	Flexible Adaptation for Opportunistic Infection .....	114
	References .....	114

### 1 Action at the "Pathogenic Cusp"

The previous chapters have discussed how phytopathogenic bacteria can sense and respond to conditions present in a variety of microenvironments: soil, water, plant cell surfaces, and intracellular spaces. The switch from epiphyte to pathogen is apparently accompanied by fundamental reprogramming of gene activity and attendant function, as evidenced by induction of *htrp* genes and subsequent production of various virulence and pathogenicity factors, some of which are host-specific, some not. This reprogramming switch between epiphytic and pathogenic growth strategies, "the pathogenic cusp" (Dangl 1994), is the point at which not only the potential pathogen but also the host first sense and respond to each other. A successful plant defense response should be based on surveillance and interfection before the pathogen has a chance to establish production of the army of factors which determine successful colonization of that host. It is incumbent on each potential plant host, then, to evolve mechanisms to recognize some factor, preferably one produced at this pathogenic cusp, and to base resistance strategies on early recognition. Thus, an evolutionary tug-of-war is

# The HrpZ Proteins of *Pseudomonas syringae* pvs. *syringae*, *glycinea*, and *tomato* Are Encoded by an Operon Containing *Yersinia* *ysc* Homologs and Elicit the Hypersensitive Response in Tomato but not Soybean

Gail Preston,<sup>1</sup> Hsiou-Chen Huang,<sup>2</sup> Sheng Yang He,<sup>3</sup> and Alan Collmer<sup>1</sup>

<sup>1</sup>Department of Plant Pathology, Cornell University, Ithaca, NY 14853 U.S.A.; <sup>2</sup>Agricultural Biotechnology Laboratories, National Chung-Hsing University, Taichung, Taiwan 40227 R.O.C.; <sup>3</sup>Department of Plant Pathology, University of Kentucky, Lexington, KY 40546 U.S.A.  
Received 6 February 1995. Accepted 18 May 1995.

The *Pseudomonas syringae* pathovars are composed of host-specific plant pathogens that characteristically elicit the defense-associated hypersensitive response (HR) in nonhost plants. *P. s. pv. syringae* 61 secretes an HR elicitor, harpin<sub>PM</sub> (HrpZ<sub>PM</sub>), in a *hrp*-dependent manner. An internal fragment of the *P. s. pv. syringae* 61 *hrpZ* gene was used to clone the *hrpZ* locus from *P. s. pv. glycinea* race 4 (bacterial blight of soybean) and *P. s. pv. tomato* DC3000 (bacterial speck of tomato). DNA sequence analysis revealed that *hrpZ* is the second ORF in a polycistronic operon. The amino acid sequence identities of HrpZ<sub>PM</sub>/HrpZ<sub>PM</sub> and HrpZ<sub>PM</sub>/HrpZ<sub>PM</sub> were 79 and 63%, respectively. Although none of the HrpZ proteins showed significant overall sequence similarity with other known proteins, HrpZ<sub>PM</sub> contained a 24-amino acid sequence that is homologous with a region of the PopA1 elicitor protein of the tomato pathogen, *Pseudomonas solanacearum* GM1000. *hrpA*, the upstream ORF, was highly divergent: The amino acid sequence identities of HrpA<sub>PM</sub>/HrpA<sub>PM</sub> and HrpA<sub>PM</sub>/HrpA<sub>PM</sub> were 91 and 28%, respectively, and no HrpA sequence showed similarity to known proteins. In contrast, the predicted products of the downstream ORFs in *P. s. pv. syringae* and *P. s. pv. tomato*, *hrpB*, *hrpC*, *hrpD*, and *hrpE* showed varying levels of similarity to those of *yscI*, *yscJ*, *yscK*, and *yscL*. These are colinearly arranged genes in the *virC* locus of *Yersinia* spp., which are involved in the secretion of the Yop virulence proteins via the type III pathway. The similarity of the Ysc proteins was generally stronger in comparisons with the *P. s. pv. tomato* Hrp proteins. The HrpZ proteins were purified by heat denaturation of contaminating proteins followed by ammonium sulfate fractionation, hydrophobic chromatography, and gel electrophoresis. All three HrpZ proteins elicited the HR in tomato, whereas none of them elicited significant necrosis in soybean. The results indicate that HrpZ is encoded in an operon containing some of the genes involved in its own secretion and suggest that HrpZ structure does not directly determine bacterial host range.

Phytopathogenic strains of *Pseudomonas syringae* cause two patterns of necrosis when the bacteria invade a plant. On a susceptible ("compatible") host, a necrotic lesion often develops over a period of days, with necrosis spreading as the bacteria multiply and the plant becomes diseased. On a resistant or nonhost plant, a localized cellular necrosis is induced within 24 to 48 h, and bacterial multiplication is inhibited. This was first reported by Klement (1963; Klement et al. 1964), who observed that when high concentrations of pathogenic bacteria are infiltrated into an incompatible plant they elicit a visible necrosis which is limited to the infiltrated area. This reaction, called the hypersensitive response (HR), involves localized cell death and production of anti-microbial compounds at the site of pathogen invasion (Bonas 1994). The ability of *P. syringae* and other nontumorigenic, gram-negative, bacterial pathogens to elicit the HR is governed by *hrp* genes. Typical Hrp<sup>-</sup> mutants are pleiotropically defective in plants: They do not elicit the HR in nonhosts and they fail to multiply and cause disease in host plants (Lindgren et al. 1986). Clusters of *hrp* genes have been identified in many gram-negative phytopathogenic bacteria (Bonas 1994). A 25-kb *hrp* cluster from *P. s. pv. syringae* 61 is sufficient to confer the tobacco HR phenotype, but not the pathogenic phenotype on nonpathogenic bacteria (Huang et al. 1988). *hrp* genes have also been cloned and characterized extensively from *P. s. pv. phaseolicola* NPS3121, *P. solanacearum* GM1000, *Xanthomonas campestris* pv. *vesicatoria* 75-3, and *Erwinia amylovora* Ea321 (Lindgren et al. 1986; Boucher et al. 1987; Beer et al. 1991; Bonas et al. 1991). Certain *hrp* genes are widely conserved among these pathogens, and several encode components of a protein secretion pathway that is similar to the type III pathway used by *Yersinia*, *Shigella*, and *Salmonella* spp. to secrete extracellular proteins involved in animal pathogenesis (Van Gijsegem et al. 1993). One activity of the *hrp*-encoded secretion pathway in phytopathogenic bacteria is the secretion of proteinaceous elicitors of the HR, which are also encoded by *hrp* genes.

The first *hrp*-encoded elicitor characterized was harpin<sub>PM</sub> from *E. amylovora* (Wei et al. 1992). Similar elicitors have since been isolated from other bacteria, including *P. s. pv. syringae* 61, *P. solanacearum* GM1000, and *E. chrysanthemi*.



EC16 (He et al. 1993; Arlat et al. 1994; Bauer et al. 1994). Proteins in this family of elicitors share several general characteristics. They are glycine rich, heat-stable, lack cysteine, and appear highly susceptible to proteolysis. They lack an N-terminal signal peptide, but they are secreted to the bacterial milieu. Their expression and secretion is dependent on *hrp* genes. The biological role of these proteins in pathogenesis has not yet been determined, but the purified proteins can induce an HR on a nonhost plant such as tobacco. However, there are significant differences in the organization of the elicitor operons and the activity of the elicitors, which suggests that the *Erwinia* harpins, the *P. syringae* *hrpZ* product and the *P. solanacearum* *popA* product may represent three distinct classes of elicitors. In this work we will refer to the *P. s. pv. syringae* elicitor as *HrpZ<sub>ps</sub>* rather than *harpin<sub>ps</sub>* (He et al. 1993). This distinction is supported by the weak similarity of the amino acid sequences of the four proteins, with the only exception being the C-terminal halves of the *Erwinia* harpins (Bauer et al. 1994).

The location of known elicitor genes in reference to the *hrp* cluster varies in *P. s. pv. syringae*, *P. solanacearum*, and *E. amylovora*. *hrpN* and *hrpZ* are contiguous or within the *hrp* cluster, whereas *popA* lies outside (although near) the *P. solanacearum* *hrp* cluster (Wei et al. 1992; He et al. 1993; Arlat et al. 1994). There are no genes downstream of the elicitor gene in either the *hrpN* or the *popA* operons, which means that mutations in the elicitor genes do not have a polar effect on the Hrp phenotype, and mutant construction is straightforward. In contrast, mutagenesis and complementation studies of the *hrp* cluster from *P. s. pv. syringae* 61 have indicated that *hrpZ* lies upstream of at least one other *hrp* gene within an operon (Huang et al. 1991; Xiao et al. 1992).

In *E. amylovora* and *E. chrysanthemi*, harpins have been demonstrated to be sufficient and necessary to elicit the HR, and mutation of *hrpN* in *E. amylovora* has shown that *harpin<sub>ps</sub>* is required for pathogenesis (Wei et al. 1992). However *hrpN* mutants of *E. chrysanthemi* can establish infections, albeit at a significantly reduced frequency, which suggests that *harpin<sub>ps</sub>* is important but not essential for pathogenesis (Bauer et al. 1995). In contrast, a *popA* mutant of *P. solanacearum* is fully pathogenic on susceptible hosts, indicating that *PopA1* is not required for pathogenesis (Arlat et al. 1994).

These elicitors may play a role in controlling the host specificity exhibited by *E. amylovora* and plant pathogenic pseudomonads such as *P. syringae* and *P. solanacearum*. However it is difficult to compare the activity of *HrpZ<sub>ps</sub>* and *harpin<sub>ps</sub>* in host and nonhost plants because legumes and rosaceous plants, the hosts of *P. s. pv. syringae* 61 and *E. amylovora* Ea321, respectively, respond poorly to preparations of any of these elicitor proteins (Wei et al. 1992; He et al. 1993). *PopA1* from *P. solanacearum* does appear to act in a host-specific manner, inducing an HR on resistant lines of petunia and the nonhost tobacco, but not on susceptible lines of petunia or tomato (Arlat et al. 1994). This phenotype is similar to that of *avr* genes, but *PopA1* is distinct from known *Avr* proteins in eliciting the HR directly on resistant plants. *Harpin<sub>ps</sub>* elicits an HR on some compatible hosts of *E. chrysanthemi*, but in contrast to the other three bacteria *E. chrysanthemi* is a broad-host range pathogen and the activity of *harpin<sub>ps</sub>* may not be representative of elicitor activity in a highly host-specific system (Bauer et al. 1995).

In previous work we cloned and characterized the *hrpZ* gene from *P. s. pv. syringae* 61, a weak pathogen of bean, and demonstrated with Southern and immunoblots that the pathovars of *P. syringae* contain homologs of this gene (He et al. 1993). This supported the hypothesis that *HrpZ* represents a family of elicitors common to all pathogenic strains of *P. syringae*. We report here the isolation of homologs of *HrpZ<sub>ps</sub>* from two other experimentally important pathovars of *P. syringae*-*P. s. pv. tomato* and *P. s. pv. glycinea*. Examining *HrpZ* from these three pathovars enabled us to look within this family of elicitors for variations in sequence and activity which could indicate a role in host range determination. In addition, we characterized the two genes flanking *hrpZ* in *P. s. pv. syringae* and *P. s. pv. glycinea* and the entire *hrpZ* operon of *P. s. pv. tomato*. In conjunction with an accompanying paper (Huang et al. 1995), this completes the sequence of the *P. s. pv. syringae* 61 *hrp* genes carried on pHIR11 and provides clues to the function of the genes downstream of *hrpZ*. A preliminary account of portions of this work has been published (Collmer et al. 1994).

## RESULTS

### Cloning *hrpZ* from *P. s. pv. tomato* and *P. s. pv. glycinea*.

We previously used Southern hybridization to demonstrate that both *P. s. pv. glycinea* race 4 and *P. s. pv. tomato* DC3000 contain sequences homologous to a 0.75 kb *BstXI* internal fragment of *hrpZ* from *P. s. pv. syringae* (He et al. 1993). The same probe was used to screen genomic libraries of *P. s. pv. glycinea* and *P. s. pv. tomato*. The libraries were constructed in *E. coli* DH5 $\alpha$  by inserting 8- to 12-kb fragments from partial *Sau3AI* digests of genomic DNA into the *BamHI* site of pUCP19. The screen identified two plasmids with inserts of approximately 10 kb: pCPP2201 (*P. s. pv. tomato*) and pCPP2200 (*P. s. pv. glycinea*). The same *BstXI* fragment was used to probe a Southern blot of pCPP2201 and pCPP2200 digested with *BamHI*, *EcoRI*, and *PstI*. The probe identified two *PstI* fragments of 2.2 and 2.4 kb from pCPP2201 and pCPP2200 respectively (Fig. 1). The two *PstI* fragments were cloned into the *PstI* site of pBluescript II SK(-) (Stratagene, La Jolla, CA) in *E. coli* DH5 $\alpha$  to create the plasmids pCPP2202 to pCPP2205, with the inserts in both orientations with respect to the *lac* promoter. Cell lysates of *E. coli* DH5 $\alpha$  containing pCPP2203 (*hrpZ<sub>ps</sub>* in the vector promoter orientation) and pCPP2202 (*hrpZ<sub>ps</sub>* in the vector promoter orientation) induced an HR on tobacco, but those from cells containing pCPP2205 (*hrpZ<sub>ps</sub>* in the opposite orientation of the vector promoter) and pCPP2204 (*hrpZ<sub>ps</sub>* in the opposite orientation of the vector promoter) did not. HR activity was retained after incubating the lysate for 10 min at 100°C and removing denatured proteins by centrifugation. Insensitivity to heat treatment is a characteristic feature of previously isolated HR elicitors. Proteins in the lysates were separated on an SDS-polyacrylamide gel, transferred to an Immobilon-P membrane and immunoblotted with antibodies raised against purified *HrpZ<sub>ps</sub>*. Cross-reacting proteins of a similar size to *HrpZ<sub>ps</sub>* were observed and provisionally named *HrpZ<sub>ps</sub>* and *HrpZ<sub>ps</sub>* (Fig. 2, lanes 2 and 4).

The intensity of the *HrpZ<sub>ps</sub>* and *HrpZ<sub>ps</sub>* bands was quite low in comparison to the band for *HrpZ<sub>ps</sub>* expressed from pSYH10 in *E. coli* DH5 $\alpha$  (Fig. 2, lane 1). This implied either

that expression was low due to the distance of the cloned gene from the *lac* promoter or that  $HrpZ_{P_{hrp}}$  and  $HrpZ_{P_{hrp}}$  did not hybridize strongly to the antibodies. A band corresponding to  $HrpZ_{P_{hrp}}$  from pSYH10 could be clearly seen on a Coomassie-stained gel, but the bands for  $HrpZ_{P_{hrp}}$  and  $HrpZ_{P_{hrp}}$  were indistinct, which implies that low expression was a primary reason for the low signal. In an attempt to improve the level of expression of  $HrpZ_{P_{hrp}}$  and  $HrpZ_{P_{hrp}}$  we subcloned *EcoRI*-*Bam*HI fragments containing the inserts from pCPP2202 and pCPP2203 behind the T7 promoter of pET21(+) in *E. coli* BL21(DE3) to create the plasmids pCPP2206 and pCPP2207.

The T7 promoter enabled a moderate improvement in protein expression (Fig. 2, lanes 3 and 5).

A common arrangement of ORFs in the *hrpZ* operons of *P. s. pv. syringae*, *P. s. pv. glycinea*, and *P. s. pv. tomato* revealed by DNA sequence analysis.

Previously, we determined the complete nucleotide sequence of *hrpZ* from *P. s. pv. syringae* by sequencing a 1.4-kb subclone of pHIR11 (a cosmid containing the entire *hrp* cluster from *P. s. pv. syringae*) (He et al. 1993). In addition, analysis of the complementation groups and transcriptional

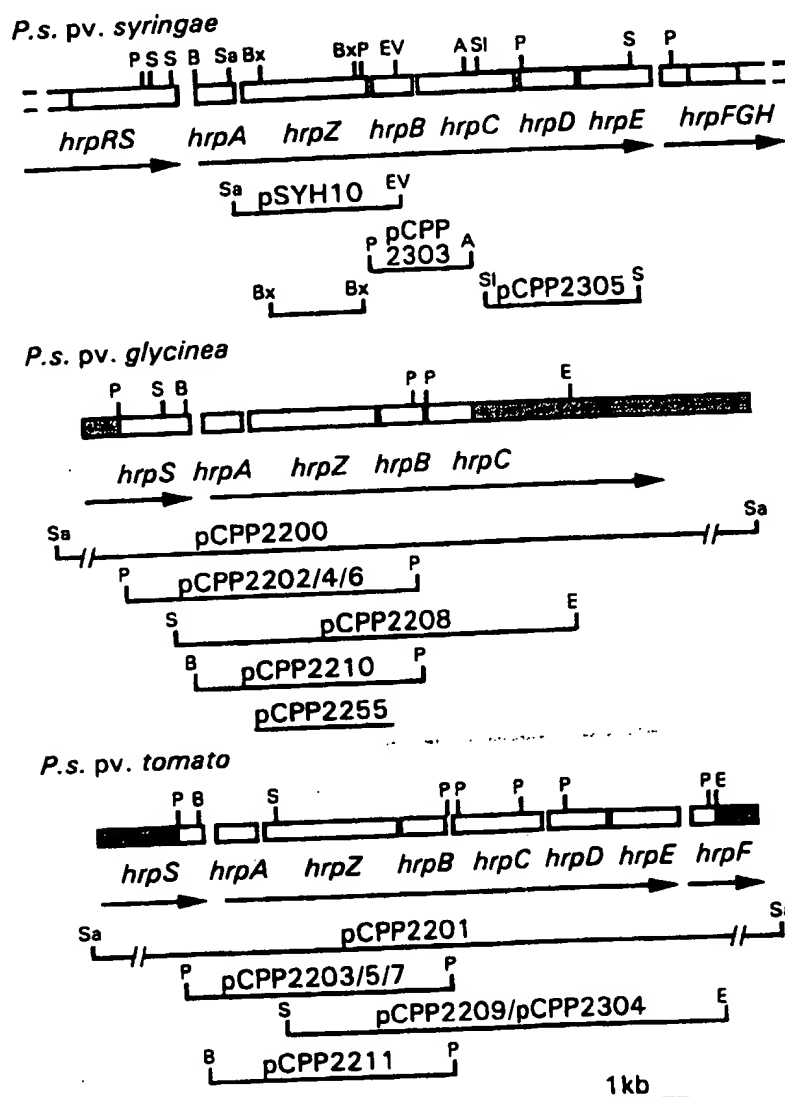


Fig. 1. Physical maps of the *hrpZ* regions from *Pseudomonas syringae* pv. *syringae* 61, *P. s. pv. glycinea* race 4, and *P. s. pv. tomato* DC3000 and clones used in this study. Open boxes represent sequenced ORFs; filled boxes represent unsequenced DNA. Direction of transcription is indicated by the arrows. Key restriction sites within the sequenced regions are indicated, along with the subclones used in this study. The 0.75-kb *Bsr*XI fragment from *hrpZ* used as a probe for *hrpZ* genes in other pathogens is also shown. Restriction endonuclease abbreviations: A, *Age*I; B, *Bgl*II; Bx, *Bsr*XI; E, *Eco*RI; EV, *Eco*RV; P, *Pst*I; S, *Sac*I; Sa, *Sau*3A; SI, *Sal*I. \* Not all sites are shown.



units of pHIR11 using Tnp $\phi$ A and Tn5-gusA1 mutagenesis (Huang et al. 1991; Xiao et al. 1992) suggested that *hrpZ* lay within an operon, upstream of at least one other *hrp* gene. Further subclones of pHIR11 were used to determine the sequence of the entire *hrpZ* operon (this study, Huang et al. 1995). We also determined the sequence of (i) the 2.2- and 2.4-kb *Pst*I subclones from pCPP2201 (*hrpZ*<sub>pm</sub>) and pCPP2200 (*hrpZ*<sub>ph</sub>), (ii) an overlapping 3.7-kb *Sac*I-*Eco*RI subclone from pCPP2201 (designated pCPP2209), and (iii) part of an overlapping 3.6-kb subclone from pCPP2200 (designated pCPP2208), as shown in Figure 1. This yielded the sequence of the entire *P. s. pv. tomato* *hrpZ* operon and the first half of the *P. s. pv. glycinea* operon. The sequenced region of *P. s. pv. syringae* and *P. s. pv. tomato* extends from *hrpS* (Xiao et al. 1994), through the *hrpZ* operon to the beginning of the *hrpH* operon (Huang et al. 1992), demonstrating that the organization of this region of the *hrp* cluster is conserved in both pathovars.

Codon preference analysis of the DNA sequence, using *P. s. pv. syringae* codon usage data, predicted that *hrpZ* was the second of six ORFs, all oriented in the same direction, an arrangement conserved in *P. s. pv. tomato* and at least the first ORFs of *P. s. pv. glycinea*. The sequence of the noncoding DNA is shown in Figure 3. Five of the six ORFs have clear potential ribosome binding sites. The fifth ORF has a putative ribosome binding site in *P. s. pv. syringae*, but the site in *P. s. pv. tomato* is less clear, the initiation codon shown being selected by alignment with the ORF in *P. s. pv. syringae*. In the absence of recognizable terminator elements downstream of the first five ORFs it seems likely that the six ORFs represent a single operon, transcribed from upstream of the first ORF. The five predicted ORFs were provisionally named *hrpA* through *hrpE*, as shown in Figures 1 and 3.

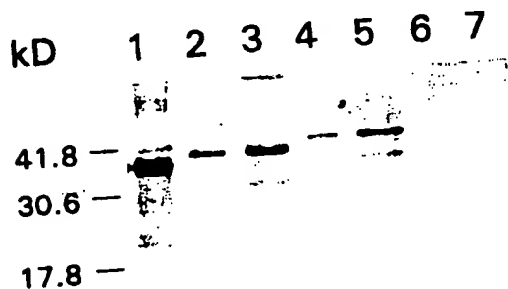


Fig. 2. Immunoblot showing expression of cloned *hrpZ* in *E. coli*. Cultures were grown in LM to an OD<sub>600</sub> of 0.8 to 1.0 at 30°C, collected by centrifugation and resuspended in 5 mM MES, pH 5.5. For lanes 3, 5 and 7, and 4, T7 expression was induced with 1 mM IPTG when the cells reached an OD<sub>600</sub> of 0.6, 3 h prior to collection. The cells were disrupted by sonication, and the crude lysate was partially purified by removal of the insoluble fraction after incubating the samples at 100°C for 10 min. SDS-loading buffer was added and the samples were incubated at 100°C for 2 min. The proteins were resolved by SDS-polyacrylamide gel electrophoresis. Following electrophoresis the proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA), probed with anti-*hrpZ* antibodies and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase. Lanes: 1, *E. coli* DH5α (pSYH10) (*hrpZ*<sub>pm</sub>); 2, *E. coli* DH5α (pCPP2202)(*hrpZ*<sub>ph</sub>); 3, *E. coli* BL21(DE3) (pCPP2206)(*hrpZ*<sub>pm</sub>); 4, *E. coli* DH5α (pCPP2203)(*hrpZ*<sub>pm</sub>); 5, *E. coli* BL21(DE3) (pCPP2207)(*hrpZ*<sub>pm</sub>); 6, *E. coli* DH5α (pBluescript II); 7, *E. coli* BL21(DE3)(pET21+).

A *hrp/avr* promoter consensus sequence lies upstream of the *hrpZ* operons of the three *P. syringae* pathovars.

The conserved sequence GGAACC—16bp—CCACNNA lies 50 bp upstream of the initiation codon of *hrpA* in all three pathovars (Fig. 3). This motif has been identified in the promoter regions of many *avr* and *hrp* genes (Innes et al. 1993; Shen and Keen 1993), and appears to be involved in positive regulation by HrpL, a putative alternative sigma factor which is itself positively regulated by HrpR and HrpS (Xiao and Hutcheson 1994). HrpL is a member of a family of alternative sigma factors, many of which are involved in secretion of extracellular factors in response to environmental stimuli (Lonetto et al. 1992). The presence of this promoter motif further supports the suggestion that the six ORFs form a single transcriptional unit which is regulated in a *hrp*-dependent manner. This motif can also be found beyond *hrpE*, upstream of *hrpFGH* in *P. s. pv. syringae* and *P. s. pv. tomato*, as indicated at the bottom of Figure 3, suggesting that the latter three ORFs form an independent *hrp*-regulated transcriptional unit in these two pathovars.

#### Comparison of the HrpZ proteins of the three *P. syringae* pathovars.

The predicted amino acid sequences for HrpZ from each of the three pathovars are aligned in Figure 4. Although the proteins migrate slightly anomalously on an SDS polyacrylamide gel, the relative sizes of the estimated molecular weights correspond to the predicted values, with HrpZ<sub>pm</sub> being the largest of the three proteins (36.5 kDa), followed by HrpZ<sub>ph</sub> (35.3 kDa) and HrpZ<sub>pm</sub> (34.7 kDa). Amino-terminal sequencing of the first 10 to 15 residues of purified HrpZ<sub>ph</sub> and HrpZ<sub>pm</sub> confirmed the predicted initiation codons of both proteins, which aligned with the start codon of HrpZ<sub>pm</sub> as shown in Figures 3 and 4. The proteins expressed in *E. coli* appear to be the same size as those recovered from the supernatants of *P. s. pv. glycinea* and *P. s. pv. tomato*, indicating that the cloned gene is intact and that there are no large post-translational modifications or deletions of HrpZ taking place in *P. syringae* but not in *E. coli*.

The amino acid sequence of HrpZ<sub>ph</sub> is quite highly conserved with respect to HrpZ<sub>pm</sub>, having 87% similarity and 79% identity. HrpZ<sub>pm</sub> is less conserved with respect to the two other proteins, with 75% similarity and 63% identity to HrpZ<sub>pm</sub>. However, the physical features of HrpZ<sub>ph</sub> and HrpZ<sub>pm</sub> are almost identical to those reported for HrpZ<sub>pm</sub> (He et al. 1993). All three are glycine-rich proteins lacking cysteine and tyrosine. HrpZ<sub>pm</sub> is the most glycine rich, being 15.7% glycine. The proteins lack the hydrophobic signal sequence used to target proteins for secretion via the Sec export pathway (Pugsley 1989). Analysis of the amino acid sequence fails to identify any obviously significant secondary structure, which is consistent with their sensitivity to proteases, and supports the suggestion that they adopt a fairly open structure in aqueous solution.

In our previous analysis of HrpZ<sub>pm</sub> (He et al. 1993), we noted the presence of two sets of short, direct repeats. Only one of these repeats, GGGLGTP, is conserved in the three proteins, with the substitution of a serine for threonine in the first repeat of both HrpZ<sub>ph</sub> and HrpZ<sub>pm</sub>. The significance of these repeats, if any, is unknown. A database search with each of the three proteins using the BLAST algorithm (Altschul et

```

syringae TTTTTCGAG AAGATCTGGA ACCGATTGCG GGACACATGC CACCTAGCTG
glycinea TTTTTCGCA. GAGCGCTGGA ACCGATTAA GGGTCGTTAC CACTA.TCTG
tomato TTTTTCGAA AGACGCTGGA ACCGTATCGC AGGCTGCTGC CACTAGTGAG

syringae TACCAAGCAA TTACGCTGGT ACAGACGAAG GGGTATGACG TTATG-----
glycinea TACCAAGCAA TTACGCTGGT ACAGACCAAG GGGTATCAGC TTATG-----
tomato TACCAAGCAA TCACGCTGGT AAATCTTAAG GGCATCAAA TCATG-----

syringae ----- --321bp----- GATTTCCTTG. ACGCCCCCTTC
glycinea ---hrpA--- --321bp----- GATTTCCTTGA ATGCCCCCAT
tomato ----- --336bp----- GATTATTTCT GATTGCCCCC
-----

syringae ATACCTGAGG GGGCTGCTAC TTTTAGGAGG TTGTG...ATG-----
glycinea CACACAGAGG GGGCTGCTAC TTTGAGGAGG TTGTG...ATG-----
tomato TCATCAGAGG GGGCCGCTAC CTTGGGATGG GCGTTTTATG-----
=> <=== ====>

syringae ----- --1020bp-----
glycinea ---hrpZ--- --1032bp-----
tomato ----- --1107bp-----

syringae -----
glycinea -----
tomato -----

syringae TACCGACAA CCGCCTGACG GAGAACTCAC DTG-----
glycinea TCACTGATAC CCGCCTGACG GAGAACTCAC GTG-----
tomato TCACTGACAG CCGCCTGACG GAGAACCACT GTG-----
-----hrpB-----

syringae --369bp----- TAGAGGTTTC CGTG-----
glycinea --369bp----- TAGAGGTTCT CGTG-----
tomato --369bp----- TAGAGGTTTC CGTG-----

syringae ----- --801bp-----
glycinea ---hrpC--- incomplete
tomato ----- --801bp-----

syringae ----- TCAATG GACCTGACCG CCGAGGACTA TTGGACTCAG
tomato ----- ATGATG AGCCTTTCTG CCGAGGATCA CTGGATTAC

syringae TGGTGGTGCA ATCCCTGGCC ATGGGCGCAT CCGGGCTGGC AAAGCCGGTT
tomato TGGTGGTGCA ACCCCTGGCA GTGGGCACAT TCGGAGTGGC ATGACCGATT

syringae CGCCGAGCGC TGCGGACTGA CCGTCAGCGA ATGTGAAGCC CTTATG-----
tomato CGCCAACGCT CGTGGGTTAT CCGTCAGTGA CTGCGATGCG CTCATG-----

syringae ----- --hrpD--- --396bp-----
tomato ----- --396bp-----

syringae ---TAGTAT. .CCGCTCCTC TCTGCACCAG GAATTCTCCC ATG-----
tomato ---TAGATCCG AACCAGCTTC TCTGCATCAG GAATACGCCC ATG-----

syringae ---hrpE--- --576bp-----
tomato ----- --576bp-----

syringae AACAGACT... C TTGCGGCGAA AATGGAACCG CTCACCTGT
tomato TACACACTCT CTGCACTCAC TGATCGCAT GATGGAACCG CTCGGCGGGT

syringae TTGCTCCACT CAAGGTTTGA ACCCTTCTGC TGGAGTATCA GGACATG
tomato TTGCTCCACT CAAGGTTTGA ACCCTTCTGC TGGAGCACCA GGACATG

```

Fig. 3. Nucleotide sequences of the noncoding regions of the *hrpZ* operon from *Pseudomonas syringae* pv. *syringae*, *P. s.* pv. *glycinea*, and *P. s.* pv. *tomato*. The sequences flanking the six ORFs of the *hrpZ* operon were aligned using the PILEUP algorithm (Genetics Computer Group). For *P. s.* pv. *syringae* and *P. s.* pv. *tomato* the sequence extends from immediately downstream of *hrpS* to the end of the operon. For *P. s.* pv. *glycinea* the sequenced region terminates at the beginning of *hrpC*. The proposed initiation and termination codons are highlighted for each ORF. The *hrp/avr* consensus sequences upstream of *hrpA* and *hrpF* are marked by double lines, with the conserved nucleotides in bold and the putative ribosome binding sites for each ORF underlined. A short inverted repeat upstream of *hrpZ* is also indicated with dashed arrows.

al. 1990) did not find significant homology to any other bacterial proteins, with the exception of a single, glycine rich region found only in HrpZ<sub>Ph</sub> (Fig. 4). This stretch of 24 amino acids has homology at both the nucleotide and amino acid level to a region of the host-specific elicitor PopA1 from *P.*

*solanacearum*, as shown at the bottom of Figure 4. There is no overall similarity of the amino acid and nucleotide sequences of HrpZ to the HR elicitors characterized from *E. amylovora*, *E. chrysanthemi*, and *P. solanacearum* except to a degree accounted for by their similar composition.

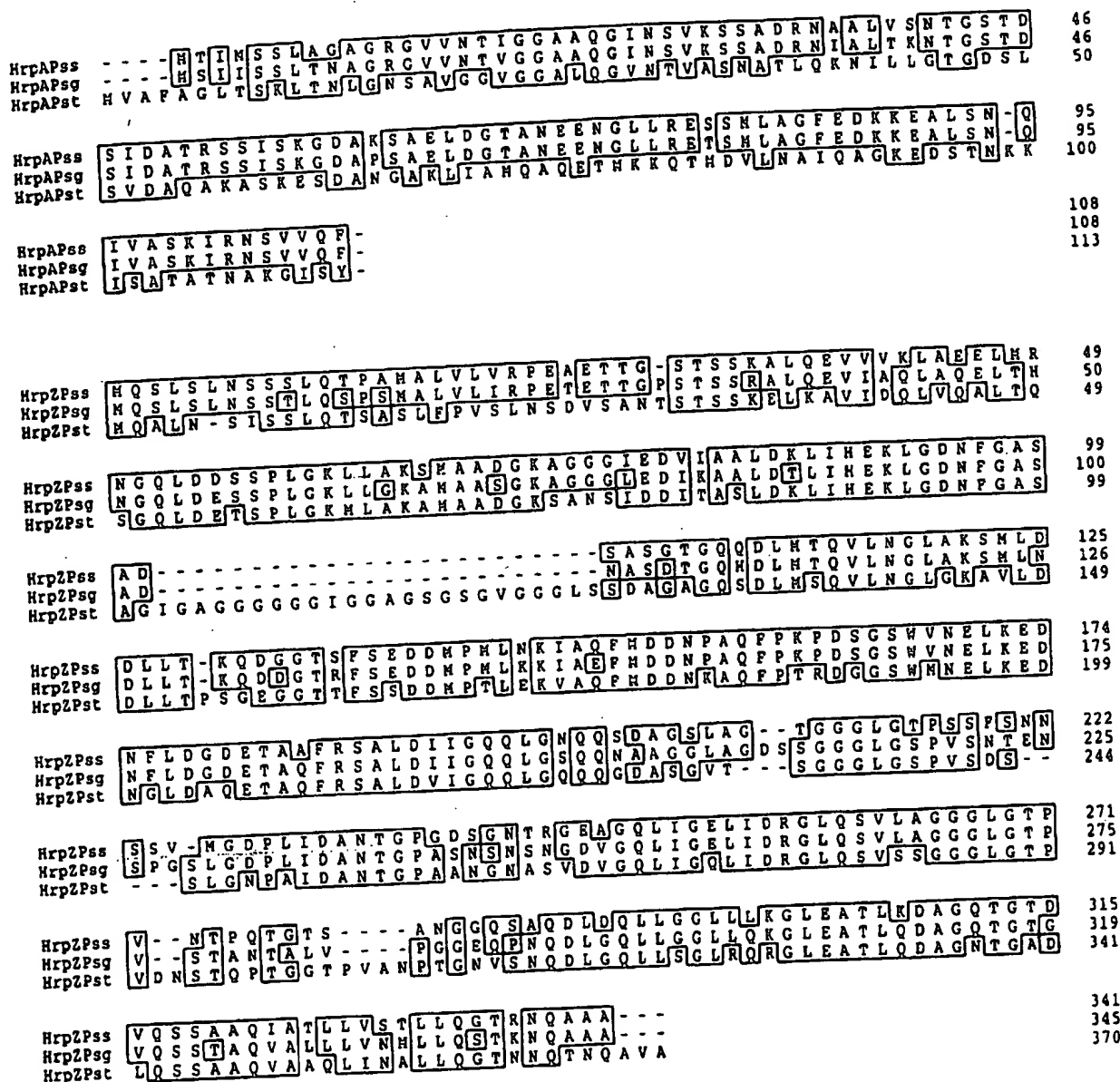


Fig. 4. Alignment of the protein sequences of HrpA and HrpZ. The predicted protein sequences of HrpA and HrpZ from *Pseudomonas syringae* pv. *syringae*, *P. s. pv. glycinea*, and *P. s. pv. tomato* were aligned using the PILEUP algorithm (Genetics Computer Group). The alignment of a unique glycine rich region of HrpZ<sub>Ph</sub> with a homologous region of PopA1 from *P. solanacearum* is also shown.

The predicted HrpA protein of *P. s. pv. tomato* differs substantially from that of *P. s. pv. syringae* and *P. s. pv. glycinea*.

The first ORF of the *hrpZ* operon starts 50 bp downstream of the conserved *hrp/avr* promoter motif, as shown in Figure 3. The predicted product is a small (11 kDa), hydrophilic protein with a hydrophobic N-terminus. An alignment of the amino acid sequences from all three pathovars is shown in Figure 4. Although the predicted sequences of HrpA from *P. s. pv. syringae* and *P. s. pv. glycinea* are highly conserved, with 92% similarity and 91% identity to each other, HrpA from *P. s. pv. tomato* is quite divergent, having only 42% similarity and 28% identity to HrpA from *P. s. pv. syringae*. The presence of a ribosome binding site and the highly conserved character of HrpA in two of the three pathovars supports the hypothesis that HrpA is translated. T7 polymerase-dependent expression of *hrpA* (described below) provides further evidence for production of a HrpA protein. Cell lysates of *E. coli* expressing only HrpA did not elicit the HR on tobacco (data not shown), which suggests that it does not contribute directly to the HR. The role of HrpA in the bacterium is unknown, and it shows no significant homology to any previously characterized proteins.

#### T7 expression studies.

To confirm the production of proteins corresponding to the two sets of newly cloned *hrpA* and *hrpZ* genes, the *Bgl*III-*Pst*I fragments from *P. s. pv. glycinea* and *P. s. pv. tomato* were subcloned into pET21(+) and the products specifically labelled by T7 promoter/polymerase-dependent expression in *E. coli* BL21(DE3) cells incubated with [<sup>35</sup>S]-methionine (Studier et al. 1990). Radiolabeled proteins in the cell lysate were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 5). Lysates of cells containing pCPP2211 displayed unique bands which corresponded well with the predicted molecular weight of HrpA (11.5 kDa) and were consistent with the previously observed mobility of HrpZ<sub>ps</sub> (Fig. 5, lane 2). Lysates of cells containing pCPP2210 contained bands corresponding to HrpZ<sub>ps</sub> (36 kDa) and HrpA (11 kDa) (Fig. 5, lane 3). No HrpB band was visible in the products of pCPP2211 (Fig. 5, lane 2), but this could potentially be attributed to the omission of cysteine, which is not required for HrpA and HrpZ synthesis, from the amino-acids added to the reaction mixture. T7 expression of HrpB was independently confirmed for both *P. s. pv. syringae* and *P. s. pv. tomato* using a 0.84-kb *Pst*I-AgeI fragment of pHIR11 and the 3.7-kb *Sac*I-EcoRI fragment from pCPP2209, subcloned into LITMUS 28 to construct the plasmids pCPP2303 and pCPP2304. T7 expression in *E. coli* BL21(DE3) cells was performed as outlined above and in Figure 5. In each case a protein of about 13 kDa was observed, which corresponds well with the predicted molecular weight of HrpB from each of the two pathovars (data not shown). In an accompanying study Huang et al. (1995) have confirmed the production of proteins corresponding to HrpC, HrpD, and HrpE from *P. s. pv. syringae* 61. The similarities between the three pathovars suggest that the equivalent ORFs in *P. s. pv. glycinea* and *P. s. pv. tomato* also encode proteins. However when we independently confirmed the production of HrpD from *P. s. pv. syringae* 61 using a 1.3-kb *Sac*I-*Sac*I subclone from pHIR11 cloned into pT7-6 (pCPP2305) our results suggested the use

of an alternative initiation codon to make a larger (21 kDa) HrpD protein (data not shown). In the absence of a strong ribosome binding site at either of the putative initiation codons, the exact size of HrpD remains uncertain.

#### The four ORFs downstream of *hrpZ* show varying similarities to *Yersinia* Ysc proteins.

The *hrpC*, *hrpD*, and *hrpE* genes downstream of *hrpZ* in *P. s. pv. syringae* 61 have been sequenced and the products identified using T7 polymerase-dependent expression (Huang et al. 1995). Two of the predicted proteins, HrpC and HrpE, were shown to be homologous to the proteins YscJ and YscL, respectively, which are encoded in the *virC* operon of *Yersinia enterocolitica* and are involved in the type III secretion pathway (Michiels et al. 1991). Homologs of YscJ have also been found in the *hrp* clusters of several other phytopathogenic bacteria, including *P. solanacearum* and *X. campestris* (Fenselau et al. 1992; Gough et al. 1992). Additional homologs are *Salmonella typhimurium* FljF and *Rhizobium fredii* NolT (Jones et al. 1989; Meinhardt et al. 1993). The same four downstream ORFs are found in *P. s. pv. tomato*, and the partial sequence of the operon from *P. s. pv. glycinea* confirms the presence of the first two of these ORFs, *hrpB* and *hrpC*, in this pathovar (Fig. 6).

HrpB is fairly conserved in all three pathovars, as shown by the alignment presented in Figure 6. It encodes a small serine-rich protein of approximately 13 kDa. BLAST searches using HrpB from either *P. s. pv. syringae* or *P. s. pv. glycinea* identified no significant homologies, but a search using HrpB from *P. s. pv. tomato* identified similarity to the *Yersinia* protein, YscI. YscI is 115 amino acids long, thus slightly shorter than HrpB (127 amino acids). *yscI* lies immediately upstream of *yscJ* in the *virC* operon, which suggests that the downstream ORFs of the *hrpZ* operon might be colinear with a region of the *virC* operon.

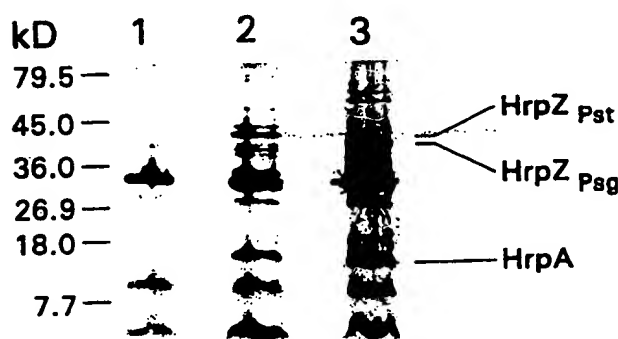


Fig. 5. T7 polymerase-dependent expression and radiolabeling of HrpA and HrpZ. T7 promoter/polymerase expression was carried out using the pET21(+) vector system in *E. coli* BL21(DE3). Cells were grown in LM to an OD<sub>600</sub> of 0.5, then centrifuged and resuspended in M9 minimal medium supplemented with 0.01% amino acids (lacking methionine and cysteine), glucose and thiamine. Cells were incubated at 30°C for 3 h and then induced with 1 mM IPTG for 10 min, followed by incubation with rifampicin at 300 µg/ml for 30 min. Cells were incubated with 10 µCi [<sup>35</sup>S]-methionine for 10 min, lysed in SDS-loading buffer, and the proteins were separated by SDS-polyacrylamide electrophoresis and visualized by autoradiography. *E. coli* BL21(DE3) cells carried the following plasmids in lanes: 1, pET21(+); 2, pCPP2211; 3, pCPP2210.

The apparent colinear arrangement of this group of *hrp* and *ysc* genes led us to inspect the *P. s. pv. syringae* and *P. s. pv. tomato* HrpD proteins for possible similarity to the *Yersinia* spp. YscK proteins. The similarity between the HrpD of *P. s. pv. syringae* and *Y. pseudotuberculosis* was the highest, with 28% of the amino acids identical and 57% similar. The HrpD and YscK proteins are of similar overall composition, and they lack any predicted transmembrane segments. However, there is a striking discrepancy between the sizes of the two proteins. HrpD is only 133 amino-acids long, whereas YscK from *Y. pseudotuberculosis* is 209 amino-acids long. From the T7 experiments described above it is important to note that in the absence of a strong ribosome binding site, the precise ini-

tiation codon of the *hrpD* ORF is uncertain; it is conceivable that *hrpD* actually initiates immediately downstream of *hrpC*, at the ATG codon which overlaps the stop codon of *hrpC*, which would yield a predicted protein of 176 amino acids for HrpZ<sub>2</sub> or 175 amino acids for HrpZ<sub>2</sub> in an arrangement similar to that of the *yscJ* and *yscK* ORFs in *Yersinia* spp. However, this codon and all other potential initiation codons upstream of the one we have chosen lack ribosome binding sites, and the pattern of codon usage suggests that the intergenic region is not translated.

Although the similarities between HrpB/YscI, HrpD/YscK, and HrpE/YscL are lower than those involving HrpC/YscJ, the similarities of HrpB/YscI and HrpE/YscL are clearly in-

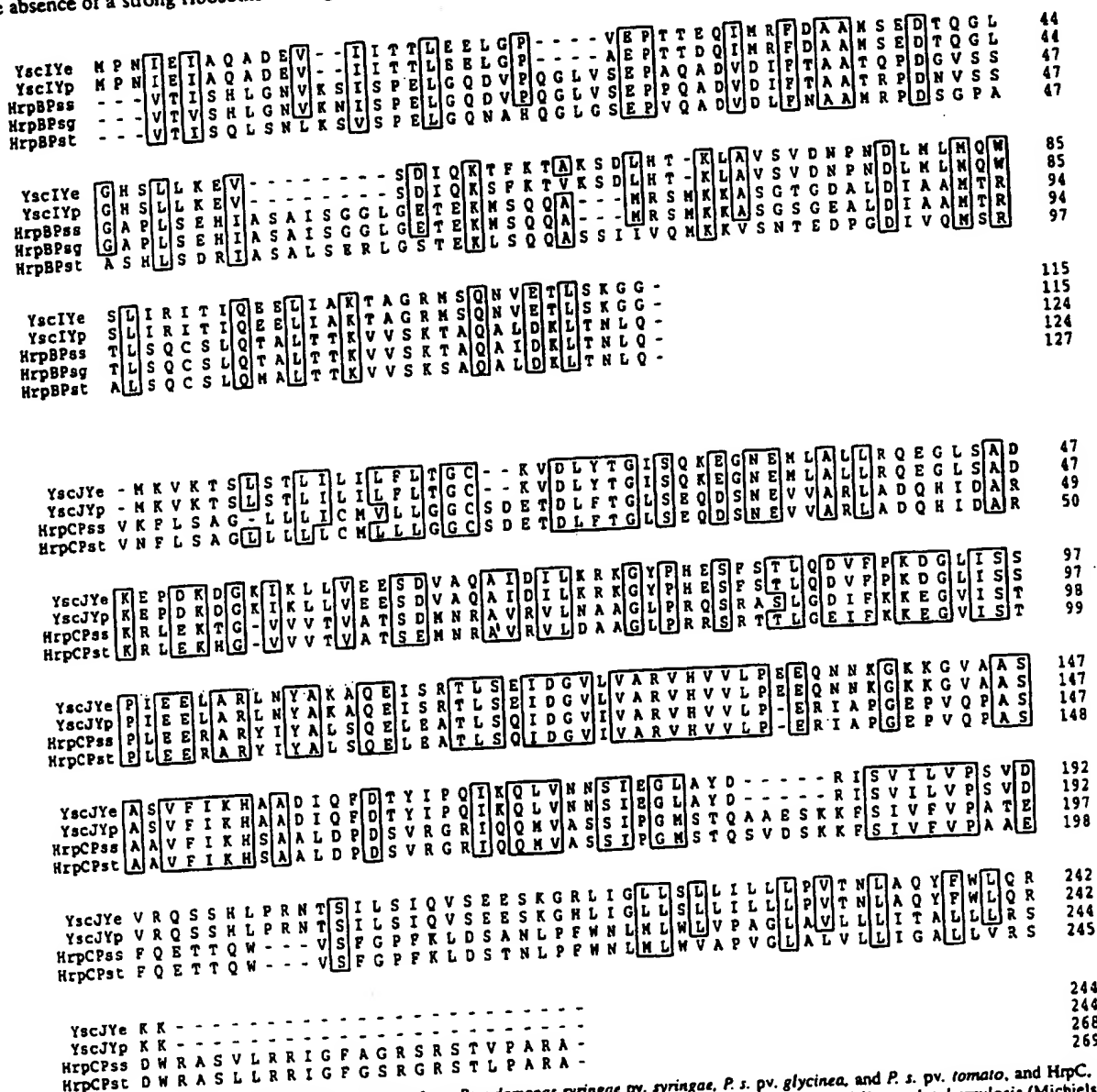


Fig. 6. Alignment of the protein sequences of HrpB from *Pseudomonas syringae* pv. *syringae*, *P. s. pv. glycinea*, and *P. s. pv. tomato*, and HrpC, HrpD and HrpE from *P. s. pv. syringae* and *P. s. pv. tomato* with YscI, YscJ, YscK, and YscL from *Y. enterocolitica* and *Y. pseudotuberculosis* (Michiels et al. 1991; Rimpilainen et al. 1992). (continued on next page)

In a recent report, Van Gijsegem et al. (1995) observe that the *P. solanacearum* GMI1000 *hrp* cluster also encodes homologs of YscJ and YscL but not YscI and YscK. It is possible that with relatively divergent Hrp sequences, similarities with Ysc proteins may be found only after examining the sequences from several plant pathogens. It is interesting to note that there is no ORF following *hrpE* that is homologous to the protein encoded by the final gene of the *virC* operon, YscM. However, the *hrpZ* operon lies immediately upstream of the *hrpH* operon (Fig. 1), and HrpH is a homolog of YscC, a secretion protein which lies upstream of *yscIJKL* within the *virC* operon (Michiels et al. 1991). This suggests that a sig-

Fig. 6. (continued from preceding page)

nificant proportion of the *virC* operon is conserved in *P. syringae*, albeit in a rearranged form. Eckhardt (1978) gels of total DNA, Southern-blotted and probed with a 0.75-kb *Bst*XI internal fragment of *hrpZ*<sub>ps</sub>, suggested that the *hrp* genes are chromosomal in the three strains of *P. syringae* studied, rather than being plasmid-borne as are the *hrp* genes of *P. solanace rum* GMI1000 or the *ysc* genes of *Yersinia* spp. (Van Gijsegem et al. 1993; data not shown). The homologies of the *hrpZ* operons are summarized in Table 1.

#### Overexpression, purification, and biological assay of HrpZ<sub>ps</sub> and HrpZ<sub>ts</sub>

Partially purified lysates of *E. coli* expressing HrpZ<sub>ps</sub> and HrpZ<sub>ts</sub> elicited a clear HR on tobacco while control lysates of *E. coli* containing vector alone did not. However the activity of the cell lysates on the two host plants was more ambiguous. Soybean is generally unreactive to cell lysates from either pathogen, while tomato is quite sensitive and sometimes weakly reactive not only to cell lysates of *E. coli* expressing HrpZ, but also to control lysates of *E. coli* containing vector alone. To accurately evaluate the biological properties of HrpZ from each of the two pathovars, it was necessary to purify HrpZ. It was also necessary to ascertain that the HR observed on tobacco was due solely to HrpZ and not to the products of either of the two flanking ORFs, HrpA and HrpB, since HrpA and a fusion protein of HrpB were being expressed in addition to HrpZ by the original *hrpZ*<sub>ps</sub> and *hrpZ*<sub>ts</sub> clones.

As a first step towards purifying HrpZ, we attempted to increase the level of expression. From the sequence of the *Pst*I clones encoding *hrpZ* it was clear that long stretches of DNA encoding *hrpA* and the 3' end of *hrpS* (1,144 bp in *hrpZ*<sub>ts</sub>, pCPP2202 and 809 bp in *hrpZ*<sub>ps</sub>, pCPP2203) separated *hrpZ* from the *lac* promoter in pBluescript II. A series of deletions of the 5' end of the *hrpZ*<sub>ps</sub> clone were constructed using the Erase-a-Base system (Promega), bringing the *lac* promoter within 100 bp of the *hrpZ* initiation codon, and removing *hrpA*. Although cell lysates expressing the deleted clones retained HR eliciting activity, they did not show a substantial increase in gene expression. Searching for an explanation for this behavior we identified a number of potential contributing

factors. The first possibility was the presence of a *cis*-acting sequence contained in the 100 bp remaining upstream of *hrpZ*<sub>ps</sub>. Using a terminator analysis program we identified a 9-bp inverted repeat located between *hrpA* and *hrpZ* (Fig. 3). Although this repeat lacks the AT-rich sequence downstream which is characteristic of many terminators, it is possible that its presence encourages premature transcription termination. Similar repeats, albeit with weaker secondary structure, can be found upstream of *hrpZ*<sub>ps</sub> and *hrpZ*<sub>ts</sub>. A second factor contributing specifically to the low expression of *hrpZ*<sub>ps</sub> may be the absence of a strong ribosome binding site. Finally, there could be factors related to the proteins themselves, such as a lack of stability.

To eliminate possible *cis*-acting sequences and to obtain clones of *hrpZ*<sub>ps</sub> and *hrpZ*<sub>ts</sub> that lack *hrpA* and *hrpB*, the *hrpZ* genes from both pathovars were amplified by PCR, directionally cloned into pBluescript II and transformed into *E. coli* DH5 $\alpha$  F' *lacI*<sup>-</sup>. We obtained significantly increased expression of HrpZ<sub>ts</sub> using the plasmid pCPP2255 (Fig. 7), but unexpectedly, overexpression of HrpZ<sub>ps</sub> appeared to be deleterious to the cells, and plasmids recovered from transformants often showed rearrangements. To maximize expression of HrpZ<sub>ps</sub> under these conditions, we introduced subclones containing the gene behind the T7 promoter of pET21(+) (Novagen, Madison, WI). Unlike the *lac* promoter, the T7 promoter is less sensitive to distance effects, and expression of HrpZ<sub>ps</sub> in *E. coli* BL21(DE3), with pET21(+) as the vector, resulted in increased expression as shown in Figures 2 and 8. Expression in BL21(DE3) also allowed us to retain almost complete repression of *hrpZ* until induction with IPTG. Good expression of HrpZ<sub>ps</sub> was achieved using the plasmid pCPP2211 in *E. coli* BL21(DE3).

The quality of the samples obtained following partial purification of the lysates by heat treatment was quite variable. To ensure removal of the majority of the contaminating proteins and to obtain a more concentrated sample of protein, we further purified HrpZ by ammonium sulphate precipitation and hydrophobic chromatography, which as indicated in Figure 8, yielded a distinct band on a Coomassie-stained gel. Purified, active HrpZ could then be obtained by electroelution from excised gel slices. This procedure was also used to isolate

Table 1. Homologies of *Pseudomonas syringae* pv. *syringae* *hrpZ* operon proteins with proteins from other *P. syringae* pathovars and *Yersinia* spp.

	HrpA (108) <sup>a</sup>	HrpZ (341)	HrpB (124)	HrpC (268)	HrpD (133) <sup>d</sup>	HrpE (193)
<i>P. s. pv. syringae</i>			(124)			
<i>P. s. pv. glycinea</i>	(108)	(345)	94/96		(133)	(193)
	91/92 <sup>b</sup>	79/87	(124)	(268)	78/87	76/87
<i>P. s. pv. tomato</i>	(108)	(370)	68/80	90/95	YscK	YscL
	28/42	63/75	YscI	YscI	(203)	(223)
<i>Y. enterocolitica</i>			(115)	(244)	26/53	21/47
			22/45 <sup>c</sup>	35/59	22/48	22/46
			24/45	38/60	(209)	(221)
			(115)	(244)	28/57	21/47
<i>Y. pseudotuberculosis</i>			22/45	35/59	23/49	22/46
			21/44	38/60		

<sup>a</sup> Number of amino acids in the protein is given in parentheses.

<sup>b</sup> Percent identical and similar amino acids in comparison with the *P. s. pv. syringae* protein.

<sup>c</sup> The first pair of values are the percent identical and similar amino acids in comparison with the *P. s. pv. syringae* protein; the second are in comparison with *P. s. pv. tomato*.

<sup>d</sup> The data presented here are for the shorter of the two potential ORFs encoding *hrpD*. The larger versions of the HrpD proteins of *P. s. pv. syringae* and *P. s. pv. tomato* would be respectively 175 and 176 amino acids long with 74/84% identity/similarity to each other.

HrpZ from the supernatants of *P. s. pv. tomato* and *P. s. pv. glycinea* grown in hrp-inducing minimal media (Fig. 9). Preparations of the purified HrpZ proteins from *P. s. pvs. syringae*, *glycinea*, and *tomato*, at a concentration of  $\geq 20 \mu\text{M}$  in MES buffer, were infiltrated into the leaves of tobacco, soybean, and tomato. The three proteins elicited a collapse involving  $>50\%$  of the infiltrated tissue in tobacco and tomato leaves that developed within 18 h and was typical of the HR elicited by incompatible *P. syringae* strains, but they caused no visible reaction in soybean. It is worth noting that tobacco and tomato plants vary substantially in their sensitivity to harpin preparations. For example, some leaves on sensitive tomato plants will respond to 2 to 5  $\mu\text{M}$  HrpZ<sub>PT</sub>, but  $\geq 20 \mu\text{M}$  is required for consistent results. Furthermore, unlike tobacco, tomato plants that have responded hypersensitively to a HrpZ preparation do not respond to subsequent infiltrations of the elicitor. The spurious necroses sometimes observed were deduced to result from mechanical damage incurred during infiltration or the infiltration of preparations contaminated with salts or containing high concentrations of vector control *E. coli* lysates. These necroses developed much more quickly (within 4 to 6 h), and were much weaker and patchier than the confluent HR elicited by HrpZ. The fact that the HR induced by HrpZ in tomato and tobacco is an active response of host tissue was confirmed by coinfiltration of either sodium vanadate at  $5 \times 10^{-3} \text{ M}$  or lanthanum chloride at  $1 \times 10^{-3} \text{ M}$ . Each of these two inhibitors of plant metabolism completely inhibited the HR elicited by HrpZ preparations from each of the three pathovars but not the necrosis caused by the other factors mentioned.

## DISCUSSION

We have used the *P. s. pv. syringae* 61 hrpZ gene to isolate the hrpZ locus from *P. s. pv. glycinea* race 4 and *P. s. pv. tomato* DC3000. Characterization of the hrpZ genes, products, and flanking DNA of these three pathovars has revealed the structure of the hrpZ operon, the relative variation among

ORFs within the operon, the presence of genes downstream of hrpZ that are colinear with a block of genes involved with *Yersinia* virulence protein secretion, and the presence in HrpZ<sub>PT</sub> of a sequence related to a sequence in the PopA1

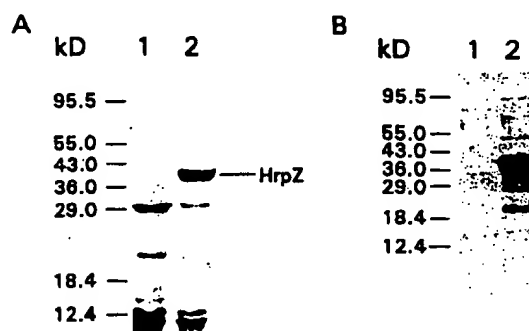


Fig. 8. Overexpression and purification of HrpZ<sub>PT</sub>. Cultures were grown to an OD<sub>600</sub> of 0.6 and induced with 1 mM IPTG. HrpZ<sub>PT</sub> was then partially purified from the cell lysate in a three-step process: first, by heat-treatment at 100°C as previously described, then by precipitation with ammonium sulphate at 30 to 45% saturation, and finally by binding to a hydrophobic resin (phenyl-sepharose) at 30% ammonium sulphate. A, Coomassie stained SDS-polyacrylamide gel. Lanes: 1, *E. coli* BL21(DE3)(pET21+); 2, *E. coli* BL21(DE3)(pCPP2211). B, Immunoblot of the samples shown in A, probed with anti-HrpZ<sub>PT</sub> antibodies and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase.

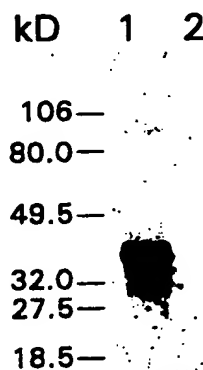


Fig. 7. Overexpression of HrpZ<sub>PT</sub> in *E. coli* DH5α F'lacI. Cultures were grown overnight at 30°C in LM with 1 mM IPTG. Cell lysates were partially purified by heat treatment, separated on an SDS-polyacrylamide gel, transferred to Immobilon-P, immunoblotted with anti-HrpZ<sub>PT</sub> antibodies, and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase. Lanes: 1, *E. coli* DH5α F'lacI (pCPP2255); 2, *E. coli* DH5α F'lacI (pBluescript II).

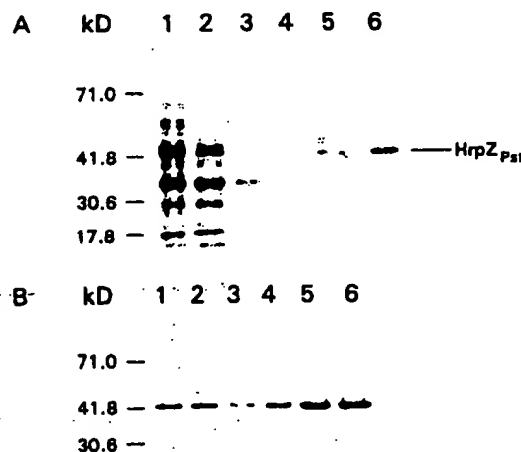


Fig. 9. Purification of HrpZ<sub>PT</sub> from hrp-induced *Pseudomonas syringae* pv. *tomato*. Cells were grown in King's broth (KB) at 30°C and then resuspended in hrp-inducing minimal medium (Huyh et al. 1989) and incubated at room temperature overnight. Cells were removed by centrifugation and the supernatant heat-treated at 100°C for 10 min. Proteins in the supernatant were precipitated with ammonium sulphate at the percent saturations indicated. Proteins were desalted, concentrated, and resuspended in 5 mM MES using Centricon-10 tubes (Amicon). A, Coomassie stained SDS-polyacrylamide gel. Lanes: 1, supernatant extracted with Strataclean resin (Stratagene); 2, heat-treated supernatant extracted with Strataclean resin (Stratagene); 3, 0 to 20% ammonium sulphate fraction; 4, 20 to 30% ammonium sulphate; 5, 30 to 40% ammonium sulphate; 6, 30 to 45% ammonium sulphate. B, Immunoblot of the samples shown in A, probed with anti-HrpZ<sub>PT</sub> antibodies and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase.



protein of the tomato pathogen *P. solanacearum* GMI1000. We also observed that purified HrpZ<sub>PopA1</sub> was at least as effective as HrpZ<sub>PopA1</sub> and HrpZ<sub>PopA1</sub> in eliciting an HR-like necrosis in the leaves of tomato, a host of *P. s. pv. tomato* DC3000, whereas none of the HrpZ preparations elicited significant necrosis in soybean, the host of *P. s. pv. glycinea*.

#### The HrpZ proteins of three *P. syringae* pathovars.

A comparison of the sequences of the three HrpZ proteins with each other and with HR elicitors characterized from other bacteria indicates that the HrpZ proteins represent a distinct family of elicitors that is conserved among *P. syringae* pathovars. The amino acid sequences of the three proteins are sufficiently similar to reveal their relatedness, but (with the exception of a sequence within HrpZ<sub>PopA1</sub>), they show no significant relatedness to elicitor proteins from other bacteria. Interestingly, *hrpZ* is the second most divergent ORF in the *hrpZ* operons of *P. s. pv. syringae* and *P. s. pv. tomato*, with only 63% of the predicted amino acids being identical. Nevertheless, HrpZ<sub>PopA1</sub>, HrpZ<sub>PopA1</sub>, and HrpZ<sub>PopA1</sub> are indistinguishable in several biological and physical properties. They have the same effect on different plants (discussed below), and they are heat stable, glycine-rich, and devoid of cysteine and tyrosine. The lack of tyrosine is a feature they differentially share with the *P. solanacearum* PopA1 protein but not the *Erwinia* harpins. This property has been speculated to allow the protein to avoid the H<sub>2</sub>O<sub>2</sub>-mediated cross-linking of tyrosine residues that may occur in plant cell walls during defense responses (Bradley et al. 1992; He et al. 1993).

Interestingly, a 24 amino acid, glycine-rich stretch of HrpZ<sub>PopA1</sub> shows homology to part of PopA1, as does the cognate nucleotide sequence. The region of homology between HrpZ<sub>PopA1</sub> and PopA1 corresponds exactly to the insertion in HrpZ<sub>PopA1</sub>. The insertion of this element within HrpZ<sub>PopA1</sub> sequences that are otherwise similar among the three HrpZ proteins suggests horizontal transfer and a common ancestry with PopA1. Because the host range of *P. solanacearum* overlaps with that of *P. s. pv. tomato*, it is tempting to speculate that this region has some particular significance to pathogenesis on tomato, although, as discussed below, this is not obvious from the different effects of the two proteins on tomato.

The presence of this insert in active HrpZ<sub>PopA1</sub> is another indicator of the apparent plasticity of structure/function relationships in these glycine-rich elicitor proteins. That significant changes to the structure of these proteins does not abolish their activity was previously demonstrated when a fortuitous *hrpZ* clone was found to produce an active derivative of HrpZ missing the N-terminal 125 amino acids, and the *popA* product was observed to be degraded in culture to an active form missing the N-terminal 93 amino acids (He et al. 1993; Arlat et al. 1994). Clearly the presence of this "additional" internal sequence does not diminish the ability of the protein to elicit the HR. In fact, although it is difficult to make a quantitative assessment, HrpZ<sub>PopA1</sub> may actually be a slightly more potent elicitor of the HR than HrpZ<sub>PopA1</sub>.

However, HrpZ<sub>PopA1</sub> appears to differ from the other HrpZ proteins in being deleterious to *E. coli* cells when overexpressed and is possibly more unstable, making it difficult to purify large amounts of the protein. Since the glycine-rich region is the most obvious difference between HrpZ<sub>PopA1</sub> and HrpZ<sub>PopA1</sub>, it is possible that it contributes to this phenomenon.

We were able to overcome this problem experimentally by using a tightly regulated T7 promoter/polymerase system, but never obtained quite the same level of expression we achieved with HrpZ<sub>PopA1</sub> and HrpZ<sub>PopA1</sub>. However, there remains the obvious question of how HrpZ toxicity is avoided by *P. s. pv. tomato*. One possibility would be that HrpZ is never expressed at levels high enough to affect the bacterium, even when it is induced in planta. Some indirect evidence for this hypothesis is provided by our examination of the DNA upstream of *hrpZ*<sub>PopA1</sub>. The ORF has a weak ribosome binding site, and we also observed that expression of cloned *hrpZ* from the *lac* promoter appears to be attenuated by the presence of *cis*-acting upstream sequences. A 9-bp GC-rich repeat upstream of *hrpZ* may be significant in this regard. Preliminary data from northern blotting experiments also indicate that premature transcription termination may take place when *hrpA*-*hrpZ* clones are expressed in *E. coli* (G. Preston, unpublished). A second possibility is that the location of the *hrpZ* gene in an operon with secretion genes ensures tight coupling of synthesis and secretion. Genes encoding extracellular proteins and secretion pathway components are often coregulated, but with a few exceptions involving the type I pathway, they do not lie within the same operon (Fath and Kolter 1993). A third possibility is that *P. s. pv. tomato* is more tolerant of high levels of HrpZ than is *E. coli*, or it possesses a means of keeping HrpZ in a nontoxic form while it is in the cell.

Further comparison with the *Yersinia* virulence system presents an intriguing possibility in this regard. It has been shown that secretion of certain "Yops" (the *Yersinia* pathogenicity determinants), involves chaperone proteins, small hydrophilic proteins which help keep the Yop protein in a translocation competent form and help target it for secretion (Wattiau et al. 1994). The genes encoding each chaperone are located adjacent to the gene encoding the corresponding Yop. Given the presence of several small ORFs of undetermined function in the pHIR11 *hrp* cluster, it is tempting to speculate that one of them, particularly *hrpA*, might encode a protein with chaperone function. There is a superficial resemblance between HrpA and *Yersinia* chaperones such as SycE. They are all small, hydrophilic, cytoplasmic proteins which lack a signal sequence, but there are no specific homologies. We are now constructing nonpolar mutations to test the role of HrpA in secretion. Preliminary results suggest that HrpA is not required for *E. coli* MC4100(pHIR11) to elicit an HR or secrete HrpZ (J. R. Alfano, unpublished), but in chaperone-mediated systems limited secretion of a protein will usually occur even in the absence of its chaperone, so it may be necessary to look quantitatively at secretion and accumulation of HrpZ to assess whether mutations in *hrpA* or other *hrp* genes have an effect.

#### The colinear relationship between several *hrp* and *ysc* genes.

From the sequence of the *hrpZ* operon it is clear that the parallels with the *Yersinia* type III secretion pathway extend beyond homologies of individual genes. The four genes downstream of *hrpZ*, *hrpB*-*E*, appear to be arranged colinearly with the region of the *virC* secretion operon from *Yersinia* that encodes YscI-L. The *virC* operon is a large operon containing 13 genes, *yscA*-*yscM*, several of which have been demonstrated to have a role in Yop secretion (Michiels et al. 1991). Of the four *Yersinia* genes with putative ho-

mologs in the *hrpZ* operon, only *yscJ* and *yscL* are known to have a role in secretion. An accompanying paper shows that five more *hrp* genes, downstream of the *hrpH* operon, are colinear with the *yscQ-U* genes in the *virB* operon of *Yersinia* (Huang et al. 1995).

It appears that a significant proportion of the type III secretion pathway described in *Yersinia* can be identified in *P. syringae*, and it seems likely that increasing parallels between the two systems will be found. In both systems the secreted proteins are involved with early events in the interaction with the host, and expression of secretion genes and virulence proteins is tightly coregulated. The secretion pathway seems to function in a similar way, as in both cases secreted proteins lack an N-terminal signal peptide and are not posttranslationally processed.

#### HrpZ and host specificity.

The function of HrpZ in compatible interactions is unclear. A likely role is the release of nutrients to the apoplast. Atkinson and Baker (1987a, 1987b) have proposed that the alkalization of the apoplast caused by Hrp<sup>+</sup> bacteria (which occurs at a slower rate in compatible interactions) results in the leakage of sucrose and other nutrients to support bacterial growth. One of the key unanswered questions regarding the *P. syringae* HrpZ proteins is their role in host specificity. Compatible interactions leading to disease are distinguished by the absence of the HR. Host-differential elicitor activity would be one way to reconcile the production of HR-eliciting proteins by *P. syringae* and the phenomenon of host-specific compatibility. The failure of the PopA1 protein to elicit the HR in tomato, a host of *P. solanacearum* GM1000, supports this concept (Arlat et al. 1994). Similarly, the isolated *P. s. pv. syringae* 61 HrpZ protein fails to elicit the HR in bean, although the significance of this is diminished by the fact that bean leaves appear insensitive to any harpins (He et al. 1993). To further explore this question, we infiltrated all three HrpZ proteins into the leaves of the host plants for each of the pathovars. The host plants of *P. s. pv. syringae* 61, and *P. s. pv. glycinea*, bean and soybean, respectively, are uniformly unresponsive to HrpZ from both compatible and incompatible pathogens; however, tomato leaves proved to be highly sensitive to all three HrpZ proteins. Thus, our data argue against the hypothesis that host-differential activity of HrpZ proteins controls the host specificity of *P. syringae* pathovars.

If isolated HrpZ<sub>61</sub> elicits the HR in tomato, why does *P. s. pv. tomato* not elicit the HR during pathogenesis? One possibility is that the response of tomato to HrpZ<sub>61</sub> is qualitatively different than the response to HrpZ<sub>61</sub> and HrpZ<sub>61</sub> despite manifestation of the same gross morphology. That is, the necrosis elicited by HrpZ<sub>61</sub> is fundamentally different than the HR and does not involve associated defenses that stop the pathogen. We are now testing this possibility with probes for HR-specific transcripts. A second possibility is that HrpZ<sub>61</sub> production is regulated in a host-specific manner. However, *hrpZ* is clearly part of the Hrp regulon: *hrpZ* expression is transcriptionally linked with genes encoding components of the secretion pathway, the *hrpZ* operons in all three of these *P. syringae* pathovars have virtually the same *hrp/avr* promoter sequence, and expression of the *hrpZ* operon is likely required for pathogenicity. The conserved promoter sequences suggests that the *hrpZ* operon is regulated in *P. s. pv. glycinea*

and *P. s. pv. tomato* by the same nutritional conditions and HrpR, HrpS, HrpL regulatory cascade described for *P. s. pv. syringae* and *P. s. pv. phaseolicola* (Grimm and Panopoulos 1989; Rahme et al. 1992; Xiao et al. 1992; Xiao et al. 1994; Xiao and Hutcheson 1994; Grimm et al. 1995). Whether differential expression of the Hrp regulon controls host specificity awaits determination. A third possibility is that the *P. syringae* pathovars produce host-specific suppressors of defense responses. This is supported by the observation that compatible pathogens do not trigger defense responses in host plants that are elicited by nonpathogens (Jakobek et al. 1993).

It is important to note that our data do not eliminate the possibility that the three HrpZ proteins actually have differential activity in host plants when delivered by living bacteria and that the HR observed may be an abnormal response resulting from the presentation of a high concentration of HrpZ in an artificial manner. In that regard, it is interesting that legumes, which appear insensitive to isolated harpins, respond to Hrp recombinant *E. coli* cells that secrete the same proteins (He et al. 1993). Experiments in which the *hrpZ* genes of *P. syringae* pathovars are switched or altered in their patterns of deployment should test more definitively the role of HrpZ in determining host specificity.

In conclusion, we have characterized an operon containing two components of the Hrp<sup>+</sup> system of *P. syringae*—a block of secretion-related genes that are conserved in eukaryotic pathogens in the genera *Pseudomonas*, *Xanthomonas*, *Erwinia*, *Yersinia*, *Shigella*, and *Salmonella* and a gene encoding an elicitor that is unique to plant pathogens. The elicitors found in the *P. syringae* pathovars are a subfamily of a larger class that appears to be characteristic of plant pathogens, and which we postulate to have a role in releasing nutrients for bacterial utilization. Our challenge now is to determine how the various components of the Hrp system have been adapted to serve plant parasitism in the face of plant defenses.

## MATERIALS AND METHODS

### Bacterial strains and plasmids.

Bacteria and plasmids used in this study are shown in Table 2. *Pseudomonads* were routinely grown in King's B broth (King et al. 1954) at 30°C, but for certain experiments the *hrp*-derepressing minimal medium of Huynh et al. (1989), adjusted to pH 5.5, was used. *E. coli* was grown in LM (Sambrook et al. 1989) or terrific broth (Tartof and Hobbs 1987). Plasmids were introduced into bacteria by transformation (Sambrook et al. 1989) or electroporation (Gene Pulser, Bio-Rad).

### Plant materials.

The plants used in this study were tobacco (*Nicotiana tabacum* L. 'Xanthii'), tomato (*Lycopersicon esculentum* Mill. 'Moneymaker'), and soybean (*Glycine max* L. 'Harosoy'). Plants were grown in a greenhouse or growth chamber at 23° to 25°C with a photoperiod of 16 to 24 h. Infiltration of plant leaves with HrpZ preparations was performed with blunt syringes as described (Huang et al. 1988).

### DNA analysis and sequencing.

All DNA manipulations, except where specified, followed standard protocols (Ausubel et al. 1987; Sambrook et al. 1989). The *hrpZ* region of pHIR11 was subcloned into

pBluescript II (Huang et al. 1995). Two *Pst*I fragments of 2.2 and 2.4 kb from pCPP2201 and pCPP2200, respectively, were subcloned into pBluescript II SK(-) in both orientations. A series of overlapping nested deletions covering both strands was generated for each of the subclones using Erase-a-Base (Promega, Madison, WI). The deletions were sequenced from double-stranded templates using Sequenase version 2.0 (U.S. Biochemicals, Cleveland, OH) and forward and reverse M13 primers. Sequencing was completed using specific primers synthesized by Integrated DNA Technologies (Coralville, IA). In addition, the 3.7 and 3.6 kb *Sac*I-*Eco*RI fragments, which overlap the *Pst*I subclones from pCPP2201 and pCPP2200, were also subcloned into pBluescript II SK(-) and sequenced using the ABI 373A DNA sequencer at the Cornell Biotechnology Program DNA sequencing facility and specific primers synthesized by IDT. Nucleotide and derived amino acid sequences were analyzed with the Genetics Computer Group Sequence Analysis Software Package (Devereaux et al. 1984). Homology searches against major sequence databases were done with the BLAST program (Altschul et al. 1990).

#### PCR amplification of *hrpZ* from *P. s. pv. glycinea* and *P. s. pv. tomato*.

The *hrpZ* genes of *P. s. pv. glycinea* and *P. s. pv. tomato* were amplified by PCR from the plasmids pCPP2202 and

pCPP2203, respectively. Reactions were performed using the PCR Optimizer kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Reactions were overlaid with mineral oil and incubated in a Hybaid Thermal Reactor (Hybaid, Teddington, U.K.) using these cycle parameters: 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 2 min at 55°C, 3 min at 72°C, followed by a final incubation of 7 min at 72°C. The primers used for *hrpZ<sub>Pa</sub>* were 5'-TACGGGATCCTTTGAGGAGGTTGTGATG-3' and 5'-TACGCTGCAGTATC AGTCAGGCAGCAGC-3', and those for *hrpZ<sub>Pt</sub>* were 5'-TACGGGATCCATGCAAGCACTTAACAGC-3' and 5'-GGAAGTGCAGCAAGCTCCGGCGATACAC-3'. All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA), and were designed to introduce a *Bam*HI and a *Pst*I site at the 5' and 3' ends, respectively, of each amplified fragment.

The *hrpZ<sub>Pa</sub>* fragment from pCPP2202 was successfully amplified in all reaction buffers tested. The *hrpZ<sub>Pt</sub>* fragment from pCPP2203 was successfully amplified using reaction buffer B (reaction concentration 60 mM Tris-HCl, 15 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgCl<sub>2</sub>, pH 8.5). PCR products of the expected sizes of 1.0 and 1.2 kb were purified from an agarose gel, digested with *Pst*I and *Bam*HI, cloned into pBluescript II, and then transformed into *E. coli* DH5 $\alpha$  F' *lac*I, yielding plasmid pCPP2255 carrying *hrpZ<sub>Pa</sub>*. Plasmids containing

Table 2. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics <sup>a</sup>	Reference or source
<i>Escherichia coli</i> DH5 $\alpha$	<i>supE44</i> $\Delta$ <i>lacU169</i> ( $\phi$ 80 <i>lacZ</i> $\Delta$ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i> <i>Nal</i> <sup>r</sup>	Hanahan 1983; Life Technologies, Inc. Grand Island, NY
DH5 $\alpha$ F' <i>lacI</i> <sup>r</sup>	F' <i>proAB</i> <sup>+</sup> <i>lacI</i> <sup>r</sup> $\Delta$ M15 <i>zcf::Tn5</i> (Km <sup>r</sup> )/ $\phi$ 80d <i>lacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> )U169 <i>endA1</i> <i>recA1</i> <i>hsdR17</i> ( <i>c<sub>H</sub>m<sub>K</sub></i> ) <sup>+</sup> <i>deoR</i> <i>thi-1</i> <i>supE44</i> <sup>+</sup> <i>gyrA96</i> <i>relA1</i>	Life Technologies Inc.
BL21(DE3)	F' <i>ompT</i> <i>hsdR<sub>B</sub></i> ( <i>c<sub>H</sub>m<sub>B</sub></i> ) <sup>+</sup> <i>dcm</i> <i>gal</i> DE3	Novagen
<i>Pseudomonas syringae</i> pv. <i>syringae</i> 61	Wild type	Baker et al. 1987
pv. <i>glycinea</i> race 4	Wild type	C. J. Baker
pv. <i>tomato</i> DC3000	Wild type, Rpf	D. E. Cuppels
Plasmids		
pBluescript II SK(-)	Cloning vector, Amp <sup>r</sup>	Stratagene
pUCP19	pUC19 derivative, Amp <sup>r</sup>	Schweizer 1991
pET21(+)	T7 transcription vector, Amp <sup>r</sup>	Novagen
pT7-6	T7 transcription vector, Amp <sup>r</sup>	Tabor and Richardson 1988
LITMUS 28	Cloning vector, Amp <sup>r</sup>	New England Biolabs
pHIR11	25-kb cosmid containing <i>P. s. pv. syringae</i> 61 <i>hrp</i> cluster	Huang et al. 1988
PSYH10	<i>hrpZ<sub>Pa</sub></i> ORF in pBluescript II	He et al. 1993
pCPP2303	0.8-kb <i>Pst</i> I- <i>Age</i> I subclone from pHIR11, containing <i>hrpB</i> , in LITMUS 28	This study
pCPP2305	1.3-kb <i>Sac</i> I- <i>Sac</i> I subclone from pHIR11, containing <i>hrpD</i> , in pT7-6	This study
pCPP2200	pUCP19 carrying 10-kb partial <i>Sau</i> 3A1 fragment of <i>P. s. pv. glycinea</i> DNA with <i>hrpZ<sub>Pt</sub></i>	This study
pCPP2202	2.4-kb <i>Pst</i> I subclone of pCPP2200 in pBluescript II; <i>hrpA<sub>Pa</sub></i> and <i>hrpZ<sub>Pa</sub></i> in expressed orientation with respect to <i>P<sub>lac</sub></i>	This study
pCPP2204	As pCPP2202 but with <i>hrpZ<sub>Pa</sub></i> in reversed orientation to <i>P<sub>lac</sub></i>	This study
pCPP2206	2.4-kb <i>Pst</i> I <i>hrpA<sub>Pa</sub></i> and <i>hrpZ<sub>Pa</sub></i> subclone from pCPP2202 in pET21(+)	This study
pCPP2208	3.6-kb <i>Sac</i> I- <i>Eco</i> RI <i>hrpZ<sub>Pa</sub></i> subclone from pCPP2200 in pBluescript II	This study
pCPP2210	1.85-kb <i>Bgl</i> II- <i>Pst</i> I <i>hrpZ<sub>Pa</sub></i> subclone from pCPP2202 in pET21(+)	This study
pCPP2255	PCR-amplified <i>hrpZ<sub>Pa</sub></i> ORF in pBluescript II	This study
pCPP2201	pUCP19 carrying 10-kb fragment of <i>P. s. pv. tomato</i> DNA with <i>hrpZ<sub>Pt</sub></i>	This study
pCPP2203	2.2-kb <i>Pst</i> I subclone of pCPP2201 in pBluescript II; <i>hrpA<sub>Pt</sub></i> and <i>hrpZ<sub>Pt</sub></i> in expressed orientation with respect to <i>P<sub>lac</sub></i>	This study
pCPP2205	As pCPP2203 but with <i>hrpZ<sub>Pt</sub></i> in reversed orientation to <i>P<sub>lac</sub></i>	This study
pCPP2207	2.2-kb <i>hrpZ<sub>Pt</sub></i> subclone from pCPP2203 in pET21(+)	This study
pCPP2209	3.7-kb <i>Sac</i> I- <i>Eco</i> RI subclone from pCPP2201 containing <i>hrpBCDE<sub>Pt</sub></i> in pBluescript II	This study
pCPP2304	3.7-kb <i>Sac</i> I- <i>Eco</i> RI subclone from pCPP2209 in LITMUS 28	This study
pCPP2211	2.0-kb <i>Bgl</i> II- <i>Pst</i> I <i>hrpZ<sub>Pt</sub></i> subclone from pCPP2203 in pET21(+)	This study

<sup>a</sup> Amp<sup>r</sup> = ampicillin resistance; Nal<sup>r</sup> = nalidixic acid resistance; Rpf<sup>r</sup> = rifampicin resistance.

PCR-amplified *hrpZ*<sub>pm</sub> were found to be unstable and appeared to promote cell lysis.

#### HrpZ purification and analysis.

HrpZ was purified from *E. coli* as previously described (He et al. 1993) with the following modifications. Cells were lysed in either 5 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.5, or cell lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0). For some experiments the supernatant from heat-treated lysate was partially purified after sonication by ammonium sulphate precipitation (25 to 45% saturation), with desalting and concentration being performed with Centricon-10 tubes (Amicon). For experiments requiring highly purified HrpZ expressed in *E. coli* BL21(DE3), the supernatant was further purified by binding to phenyl-sepharose (Sigma) in the presence of ammonium sulphate (>30% saturation) and elution with 5 mM MES, pH 5.5, followed by electrophoresis through a native 15% polyacrylamide gel. The purified protein was then eluted from excised gel slices using an Elutrap apparatus (Schleicher & Schuell) or from crushed gel slices using a Micropure separator (Amicon). Protein concentrations were determined using Bio-Rad protein assay solution. HrpZ was also purified from heat-treated supernatants of *P. syringae* grown in *hrp*-inducing medium (Huynh et al. 1989) by ammonium sulphate precipitation (25 to 45% saturation) and desalting/concentration using Centricon-10 tubes. For infiltration into plant tissue, HrpZ preparations were diluted to various degrees with 5mM MES, pH 5.5. The amino-terminal sequence analyses were performed at the Cornell Biotechnology Program Protein Analysis Facility (HrpZ<sub>pm</sub>) and the University of Kentucky Macromolecule Structure Analysis Facility (HrpZ<sub>pm</sub>).

#### T7 expression and labeling of proteins in *E. coli*.

Proteins encoded by the *hrpZ* operon were expressed in *E. coli* BL21(DE3) by using the pET21(+) T7 expression system (Novagen). Conditions for isopropyl-β-D-thiogalactopyranoside (IPTG) induction of T7 RNA polymerase-dependent expression and labeling with L-[<sup>35</sup>S]methionine were as described by Studier et al. (1990). After being labeled, cells were collected by centrifugation and then resuspended and lysed in SDS-loading buffer and the proteins resolved on an SDS-polyacrylamide gel. Gels were stained, dried and exposed to Kodak X-ray film.

#### Nucleotide sequence accession numbers.

The nucleotide sequences reported in this paper have been deposited in GenBank under accession numbers L41861 (*P. syringae* pv. *tomato* *hrpA*, *hrpZ*, *hrpB*, *hrpC*, *hrpD*, *hrpE*), L41862 (*P. syringae* pv. *glycinea* *hrpA*, *hrpZ*, *hrpB*), L41863 (*P. syringae* pv. *syringae* *hrpA*), and L41864 (*P. syringae* pv. *syringae* *hrpB*).

#### ACKNOWLEDGMENTS

We thank Kent Loeffler for the photography and Frédérique Van Gijsegem and Christian Boucher for sending us their manuscript before publication. This work was supported by NSF grant MCB 9305178.

#### LITERATURE CITED

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Arlat, M., Van Gijsegem, F., Huet, J. C., Pernollet, J. C., and Boucher, C. A. 1994. PopA1, a protein which induces a hypersensitive-like response on specific *Petunia* genotypes, is secreted via the Hrp pathway of *Pseudomonas solanacearum*. *EMBO J.* 13:543-553.
- Atkinson, M. M., and Baker, C. J. 1987. Association of host plasma membrane K<sup>+</sup>/H<sup>+</sup> exchange with multiplication of *Pseudomonas syringae* pv. *syringae* in *Phaseolus vulgaris*. *Phytopathology* 77:1273-1279.
- Atkinson, M. M., and Baker, C. J. 1987. Alteration of plasmalemma sucrose transport in *Phaseolus vulgaris* by *Pseudomonas syringae* pv. *syringae* and its association with K<sup>+</sup>/H<sup>+</sup> exchange. *Phytopathology* 77:1573-1578.
- Ausubel, F. M., Brent, R., Kingston, R., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 1987. *Current Protocols in Molecular Biology*. John Wiley & Sons, New York.
- Baker, C. J., Atkinson, M. M., and Collmer, A. 1987. Concurrent loss in Tn5 mutants of *Pseudomonas syringae* pv. *syringae* of the ability to induce the hypersensitive response and host plasma membrane K<sup>+</sup>/H<sup>+</sup> exchange in tobacco. *Phytopathology* 77:1268-1272.
- Bauer, D. W., Wei, Z.-M., Beer, S. V., and Collmer, A. 1995. The *Erwinia chrysanthemi* EC16 harpin<sub>ec</sub>: A protein that is required for elicitation of the hypersensitive response and full virulence. *Mol. Plant-Microbe Interact.* 8:484-491.
- Beer, S. V., Bauer, D. W., Jiang, X. H., Laby, R. J., Sneath, B. J., Wei, Z.-M., Wilcox, D. A., and Zumoff, C. H. 1991. The *hrp* gene cluster of *Erwinia amylovora*. Pages 53-60 in: *Advances in Molecular Genetics of Plant-Microbe Interactions*. H. Hennecke and D. P. S. Verma, ed. Kluwer Academic Publishers, Dordrecht.
- Bonas, U. 1994. *hrp* genes of phytopathogenic bacteria. Pages 79-98 in: *Current Topics in Microbiology and Immunology*, Vol. 192: Bacterial Pathogenesis of Plants and Animals—Molecular and Cellular Mechanisms. J. L. Dangl, ed. Springer-Verlag, Berlin.
- Bonas, U., Schulte, R., Fenselau, S., Minsavage, G. V., Staskiewicz, B. J., and Stall, R. E. 1991. Isolation of a gene cluster from *Xanthomonas campestris* pv. *vesicatoria* that determines pathogenicity and the hypersensitive response on pepper and tomato. *Mol. Plant-Microbe Interact.* 4:81-88.
- Boucher, C. A., Van Gijsegem, F., Barberis, P. A., Arlat, M., and Zischek, C. 1987. *Pseudomonas solanacearum* genes controlling both pathogenicity on tomato and hypersensitivity on tobacco are clustered. *J. Bacteriol.* 169:5626-5632.
- Bradley, D. J., Kjelvum, P., and Lamb, C. J. 1992. Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. *Cell* 70:21-30.
- Collmer, A., Bauer, D. W., Alfano, J. R., Preston, G., Loniello, A. O., Huang, H.-C., and He, S. Y. 1994. The role of *Pseudomonas syringae* and *Erwinia chrysanthemi* *hrp* gene products in plant interactions. Pages 49-56 in: *Advances in Molecular Genetics of Plant-Microbe Interactions*, Vol. 3. M. J. Daniels, ed. Kluwer, Dordrecht.
- Devereaux, J., Haeblerli, P., and Smithies, O. 1984. A comprehensive set of sequence analysis programs for the VAX. *Gene* 12:387-395.
- Doolittle, R. F. 1986. Of Urfs and Orfs: A primer on how to analyze derived amino acid sequences. Mill Valley, CA.
- Eckhardt, T. 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. *Plasmid* 1:584-588.
- Fath, M. J., and Kolter, R. 1993. ABC transporters: Bacterial exporters. *Microbiol. Rev.* 57:995-1017.
- Fenselau, S., Balbo, I., and Bonas, U. 1992. Determinants of pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. *Mol. Plant-Microbe Interact.* 5:390-396.
- Gough, C. L., Genin, S., Zischek, C., and Boucher, C. A. 1992. *hrp* genes of *Pseudomonas solanacearum* are homologous to pathogenicity determinants of animal pathogenic bacteria and are conserved among plant pathogenic bacteria. *Mol. Plant-Microbe Interact.* 5:384-389.
- Grimm, C., and Panopoulos, N. J. 1989. The predicted protein product of a pathogenicity locus from *Pseudomonas syringae* pv. *phaseolicola* is homologous to a highly conserved domain of several procaryotic regulatory proteins. *J. Bacteriol.* 171:5031-5038.

- Grimm, G., Aufsatz, W., and Panopoulos, N. J. 1995. The *hrpRS* locus of *Pseudomonas syringae* pv. *phaseolicola* constitutes a complex regulatory unit. *Mol. Microbiol.* 15:155-165.
- Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166:557-580.
- He, S. Y., Huang, H.-C., and Collmer, A. 1993. *Pseudomonas syringae* pv. *syringae* harpin<sub>ps</sub>: A protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. *Cell* 73:1255-1266.
- Huang, H.-C., He, S. Y., Bauer, D. W., and Collmer, A. 1992. The *Pseudomonas syringae* pv. *syringae* 61 *hrpH* product: An envelope protein required for elicitation of the hypersensitive response in plants. *J. Bacteriol.* 174:6878-6885.
- Huang, H.-C., Hutcheson, S. W., and Collmer, A. 1991. Characterization of the *hrp* cluster from *Pseudomonas syringae* pv. *syringae* 61 and *TaphoA* tagging of genes encoding exported or membrane-spanning Hrp proteins. *Mol. Plant-Microbe Interact.* 4:469-476.
- Huang, H.-C., Lin, R.-W., Chang, C.-J., Collmer, A., and Deng, W.-L. 1995. The complete *hrp* gene cluster of *Pseudomonas syringae* pv. *syringae* 61 includes two blocks of genes required for harpin<sub>ps</sub> secretion that are arranged collinearly with *Yersinia* *ycs* homologs. *Mol. Plant-Microbe Interact.* 8:733-746.
- Huang, H.-C., Schuurink, R., Denny, T. P., Atkinson, M. M., Baker, C. J., Yucel, I., Hutcheson, S. W., and Collmer, A. 1988. Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco. *J. Bacteriol.* 170:4748-4756.
- Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. 1989. Bacterial blight of soybean: Regulation of a pathogen gene determining host cultivar specificity. *Science* 245:1374-1377.
- Innes, R. W., Bent, A. F., Kunkel, B. N., Bisgrove, S. R., and Staskawicz, B. J. 1993. Molecular analysis of avirulence gene *avrRpt2* and identification of a putative regulatory sequence common to all known *Pseudomonas syringae* avirulence genes. *J. Bacteriol.* 175:4859-4869.
- Jakobek, J. L., Smith, J. A., and Lindgren, P. B. 1993. Suppression of bean defense responses by *Pseudomonas syringae*. *Plant Cell* 5:57-63.
- Jones, C. J., Homma, M., and Macnab, R. M. 1989. L-, P-, and M-ring proteins of the flagellar basal body of *Salmonella typhimurium*: Gene sequences and deduced protein sequences. *J. Bacteriol.* 171:3890-3900.
- King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Med.* 22:301-307.
- Klement, Z. 1963. Rapid detection of pathogenicity of phytopathogenic pseudomonads. *Nature* 199:299-300.
- Klement, Z., Farkas, G. L., and Lovrekovich, L. 1964. Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. *Phytopathology* 54:474-477.
- Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. 1986. Gene cluster of *Pseudomonas syringae* pv. "*phaseolicola*" controls pathogenicity of bean plants and hypersensitivity on nonhost plants. *J. Bacteriol.* 168:512-522.
- Lonetto, M., Gribkov, M., and Gross, C. A. 1992. The  $\sigma^{70}$  family: Sequence conservation and evolutionary relationships. *J. Bacteriol.* 174:3843-3849.
- Meinhardt, L. W., Krishnan, H. B., Balati, P. A., and Pueppke, S. G. 1993. Molecular cloning and characterization of a *sym* plasmid locus that regulates cultivar-specific nodulation of soybean by *Rhizobium fredii* USDA257. *Mol. Microbiol.* 9:17-29.
- Michiels, T., Vanooteghem, J.-C., de Rouvroit, C. L., China, B., Gustin, A., Boudry, P., and Cornelis, G. R. 1991. Analysis of *virC*, an operon involved in the secretion of Yop proteins by *Yersinia enterocolitica*. *J. Bacteriol.* 173:4994-5009.
- Pugsley, A. P. 1989. Protein Targeting. Academic Press, New York.
- Rahme, L. G., Mindrinos, M. N., and Panopoulos, N. J. 1992. Plant and environmental sensory signals control the expression of *hrp* genes in *Pseudomonas syringae* pv. *phaseolicola*. *J. Bacteriol.* 174:3499-3507.
- Rimpilainen, M., Forsberg, A., and Wolf-Watz, H. 1992. A novel protein, LcrQ, involved in the low-calcium response of *Yersinia pseudotuberculosis* shows extensive homology to YopH. *J. Bacteriol.* 174:3355-3363.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning. A Laboratory Manual, Second Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schweizer, H. P. 1991. *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. *Gene* 97:109-112.
- Shen, H., and Keen, N. T. 1993. Characterization of the promoter of avirulence gene D from *Pseudomonas syringae* pv. *tomato*. *J. Bacteriol.* 175:5916-5924.
- Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 185:60-89.
- Tabor, S., and Richardson, C. C. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. USA* 82:1074-1078.
- Tartof, K. D., and Hobbs, C. A. 1987. Improved media for growing plasmid and cosmid clones. *Bethesda Res. Lab. Focus* 9:12.
- Van Gijsegem, F., Genin, S., and Boucher, C. 1993. Evolutionary conservation of pathogenicity determinants among plant and animal pathogenic bacteria. *Trends Microbiol.* 1:175-180.
- Van Gijsegem, F., Gough, C., Zischek, C., Niqueux, E., Arlat, M., Genin, S., Barberis, P., German, S., Castello, P., and Boucher, C. 1995. The *hrp* gene locus of *Pseudomonas solanacearum*, which controls the production of a type III secretion system, encodes eight proteins related to components of the bacterial flagellar biogenesis complex. *Mol. Microbiol.* 15:1095-1114.
- Wattiau, P., Bernier, B., Deslee, P., Michiels, T., and Cornelis, G. R. 1994. Individual chaperones required for Yop secretion by *Yersinia*. *Proc. Natl. Acad. Sci. USA* 91:10493-10497.
- Wei, Z.-M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A., and Beer, S. V. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* 257:85-88.
- Xiao, Y., Hue, S., Yi, J., Lu, Y., and Hutcheson, S. W. 1994. Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* and *hrmA* genes. *J. Bacteriol.* 176:1025-1036.
- Xiao, Y., and Hutcheson, S. W. 1994. A single promoter sequence recognized by a newly identified alternate sigma factor directs expression of pathogenicity and host range determinants in *Pseudomonas syringae*. *J. Bacteriol.* 176:3089-3091. Author's correction. 176:6158.
- Xiao, Y., Lu, Y., Heu, S., and Hutcheson, S. W. 1992. Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 *hrp* cluster. *J. Bacteriol.* 174:1734-1741.